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Assessing the antimicrobial activity of silver nanoparticles produced by *Enterobacter xiangfangensis* Pb204.

A dissertation submitted to the Faculty of Science,

University of Johannesburg

In partial fulfilment of the requirement for award of a

Master of Science Degree in Biotechnology

By

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March 2021

Declaration

I, Mathome Onismus Moeng, hereby declare that the research dissertation titled "Assessing the antimicrobial activity of silver nanoparticles produced by *Enterobacter xiangfangensis* Pb204" herewith submitted for the Master of Science (MSc) in Biotechnology at the University of Johannesburg represents my own work and that it has not been previously submitted by me to the University of Johannesburg or any other institution in application for a degree, diploma or any other qualification. I also confirm that I know what plagiarism entails and further declare that the work presented in this research is my own and other sources used have been cited and fully referenced.

Student signature: Date: 09 March 2021

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Abstract

Nanoparticles are essential materials in modern technology due to their unique properties, compatibility with biological systems and wide application particularly in biomedical applications. Silver nanoparticles (Ag-NPs) have been used extensively due to their different characteristics such as surface plasmon resonance, chemical stability, and antimicrobial activity. Antimicrobial resistance (AMR) has increased due to the misuse of antibiotics particularly in developing and undeveloped countries where there are no proper dispensing criteria. Bacteria have acquired resistance genes, rendering most antibiotics ineffective especially in immunocompromised patients. Silver nanoparticles may present a favourable alternative in combating bacteria resistant to antibiotics. Biological synthesis has been identified as the most suitable approach for nanoparticle synthesis as it provides control over size, shape, distribution and is cost effective in contrast to chemical and physical approaches. The study aimed to optimise reaction parameters for synthesis of biological Ag-NPs using *Enterobacter xiangfangensis* Pb204 and determine their antimicrobial activity against eight common human pathogens known to contaminate water sources. A cell-free extract from an overnight culture of *E. xiangfangensis* Pb204 was added to 1 mM $AgNO_3$ for Ag-NPs synthesis. Reaction pH (3, 5, 7 and 9), temperature (25 °C, 30 °C and 37 °C) and time (24 and 48 h) were evaluated during the synthesis of Ag-NPs which were characterised with the use of transmission electron microscopy (TEM) and energy dispersive x-ray (EDX) analysis. Silver nanoparticles obtained under pH 3, 5 and 9 had shape and size variation and were highly agglomerated which may affect their antimicrobial activity compared to Ag-NPs obtained under pH 7. Temperature and reaction time influenced the size, quantity of nanoparticles and reduction rate of metal ions into nanoparticles. It was then established that nanoparticles produced under the optimum conditions of pH 7, temperature of 37 °C and 24 hours incubation were stable, small, uniform-sized, spherical and well distributed. Six antibiotics (meropenem, ampicillin, cloxacillin, ciprofloxacin, vancomycin and combination-type penicillin amoxicillin and clavulanic acid) were tested in triplicate using the Kirby-Bauer method against the 8 bacterial strains for 24 and 48 hours at 35 ºC after which zones of inhibition were measured. The isolates were classified as susceptible (S), intermediate (I) or resistant (R) according to the Clinical and Laboratory Standards Institute (CLSI). Growth inhibition was strain dependent for some antibiotics whereas the antibiotic cloxacillin was ineffective on all eight strains. Ciprofloxacin was the most effective antibiotic showing activity on seven out of the eight strains tested at 5 µg dosage. The antimicrobial activity of the Ag-NPs with an average size of 16.7 ± 9 nm was also evaluated against the bacterial strains. The nanoparticles were diluted to a final concentration of 0.1 mM (10.8 µg/ml), 0.25 mM (27 µg/ml), 0.5 mM (53.9 µg/ml) and 1.0 mM (107.85 µg/ml) per well and incubated with bacterial culture in Luria Bertani (LB) broth for 24 hours at 35 ºC. Growth was monitored in duplicate by measuring the absorbance at 600 nm using an xMark Microplate spectrophotometer. Silver nanoparticles at 0.1 and 0.25 mM concentrations were unable to inhibit the growth of the bacteria as compared to those at 0.5 and 1.0 mM concentrations which were bacteriostatic. Silver nanoparticles were more effective in preventing growth of *E. faecium*, *A. baumannii*, *E. cloacae* and *V. cholerae* at a dosage of 10.79 µg Ag-NPs when

compared to the highest dosage of antibiotic for which each strain was susceptible to. The minimum concentration of Ag-NPs required to inhibit microbial growth is higher when compared to other studies. However, the dose is lower when compared to the antibiotics which were also strain dependent. The study had some limitations in terms of the methodologies which may have influenced the outcomes such as nanoparticle yield and uniformity, antimicrobial properties and a true comparison of Ag-NPs versus antibiotics. Further studies should be done to explore Ag-NPs as an alternative treatment for infections including a combination of Ag-NPs and antibiotics which may yield better results.

***Keywords*:** Ag-NPs, antibiotics, antimicrobial resistance, bacteria, biological synthesis, ESKAPE pathogens.

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Dedication

In

Loving Memories Of My Uncle And Cousin

Mamphodi Alfred Diale (1970-2011)

And

Victor Tiroane Kgaditse (1987-2018)

May Their Souls Rest In Everlasting Peace.

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“Keep faith and never lose hope. Trust that your prayers have been received by the God of Mount Zion (His Grace Bishop Dr. BE. Lekganyane).” Firstly, let me thank God of Mount Zion for life, grace, blessings and the people He brought into my life. I would like to thank myself for doing the work, for overcoming doubts and negative energy, for believing in myself and all I have done to make this study a success.

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“Instead of going with the flow, we are going to be the flow. Instead of demanding change, we are going to be the change we want to see. We are the ones we have been waiting for (Dakalo Mulima).”

Publications and conferences

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List of abbreviations

ABC	ATP Binding Cassette
Ag-NPs	Silver nanoparticles
AMR	Antimicrobial resistance
AuNPs	Gold nanoparticles
C	Carbon
CLSI	Clinical and Laboratory Standards Institute
Co	Cobalt
CRAB	Carbapenem-resistant *A. baumannii*
Cu	Copper
CVC	Central venous catheterizations
Dnr	Daunorubicin
Dox	Doxorubicin
EBV	Epstein-Barr virus
ECDC	European Centre for Disease Prevention and Control
EDX	Energy dispersive x-ray
EMEA	European Medicines Agency
ESBLs	Extended-spectrum β-lactamases
EPA	Environmental Protection Agency
EU	European Union
FDA	Food and Drug Administration
GDP	Gross domestic product
HSV	Herpex simplex virus
HIV	Human immunodeficiency virus
ICUs	Intensive care units
ICEs	Integrative and conjugated elements
I	Intermediate
KSHV	Kaposi's sarcoma-associated herpesvirus
LB	Luria Bertani
MIC	Minimum inhibitory concentration

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MLS	Macrolides, Lincosamides and Streptogramins
Mn	Manganese
MDR	Multi-drug resistant
MRSA	Methicillin resistant *Staphylococcus aureus*
NDoH	National Department of Health
Ni	Nickel
NPD	Nanotechnology Products Database
NS	Nonsusceptible
Pb	Lead
PEN	Project on Emerging Nanotechnologies
ROS	Reactive oxygen species
R	Resistant
S	Susceptible
SAASP	South African Antibiotic Stewardship Programme
sdH_2O	Sterile distilled water
SERS	Surface-enhanced Raman scattering
SIAA	Society of Industrial-Technology for Antimicrobial Articles
SSD	Susceptible-dose dependent
SU	Standard Units
TB	Tuberculosis
TEM	Transmission electron microscopy
UTIs	Urinary tract infections
VRE	Vancomycin resistant enterococci
VISA	Vancomycin intermediate *S. aureus*
VRSA	Vancomycin resistant *S. aureus*
XDR	Extremely-drug resistant
WHO	World health organization
Zn	Zinc

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List of units

ºC	Degree Celsius
h	Hour
keV	Kiloelectron volt
kV	Kilo volt
µg	Microgram
µg/ml	Microgram/milliliter
µl	Microliter
mM	Millimolar
mm	Millimeter
nm	Nanometer
OD	Optical density
ppm	Parts per million
v/v	Volume/volume
x g	Centrifugal force

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Dissertation outline

Chapter 1 outlines the introduction of the study briefly, covering the focal area of the research and highlighting the problem statement and the aim and objectives.

Chapter 2 covers the in depth review of relevant literature concerning the impacts of antimicrobial resistance (AMR) and a discussion on the use of biological Ag-NPs as an alternative treatment to combat the problems associated with AMR.

Chapter 3 describes the optimization of synthesis parameters to achieve small spherical Ag-NPs specifically referring to pH, temperature and reaction time.

Chapter 4 entails the antimicrobial profiles of Ag-NPs and antibiotics at low dosages on eight clinical pathogens which includes six ESKAPE pathogens. Comparisons are made to determine which antimicrobials are more effective based on the results obtained from the study.

Chapter 5 summarizes the research findings while drawing suitable conclusions and providing recommendations for future work.

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Chapter 1: Introduction

1.1 Background

Rickerby and Morrison (2006) reported the influence of nanoscience and nanotechnology towards sustainable development in the future, mainly affecting industries such as agri-food, healthcare, transport and energy, materials, and information and communications technologies. This is because many sectors depend on fossil fuels for energy and transport which their by-products and waste from manufacturers negatively affect the environment leading to damage of the ecosystems (Rickerby and Morrison, 2006). Almost 10 years later, Iravani (2014), reported that nanoscience and nanotechnology has gained much interest considerably because of the effect they may have on numerous science areas including pharmaceutical industries, energy, electronics and medicine. Nanoscience and nanotechnology account for nano-sized structures and materials of less than 100 nm. Manufacturing of nanomaterials with potentially unique and size-related physico-chemical properties highly different from large materials has made nanotechnology grow rapidly (Tran *et al.*, 2013). Nanomaterial based technologies have been utilized in various areas from medicine to chemistry because shape, size and composition of metal nanomaterials are closely linked to physical, chemical and optical properties (Millstone *et al.*, 2009; Lee *et al*., 2012; Lee *et al.*, 2018).

Recent research has been focusing on the biological synthesis of metal nanomaterials which makes use of biodegradable materials, moving away from traditional chemical methods employing toxic chemicals. Biosynthesis methods for nanomaterials are considered to be environmentally friendly and feasible in terms of costs. This is because they are rooted on green chemistry principles and they can easily be augmented for large production (Mohanpuria *et al*., 2007; Iravani 2011; Prabhu and Poulose 2012). Whilst the use of chemical and physical methods is more popular in nanoparticle synthesis, using toxic chemicals limits their biomedical applications, particularly in clinical fields (Li *et al.*, 2011). Applications of metal nanoparticles are common in biosensing, biological and biomedical fields with an interest to develop more eco-friendly synthesis methods (Park *et al*., 2015). Metal nanoparticles include gold, silver, platinum, palladium, quantum dots, magnetite and uraninite to mention a few. Biological synthesis of such metal nanoparticles using bacteria, yeasts, fungi, actinomycetes and viruses has been reviewed by numerous studies (Narayanan and Sakthivel, 2010).

Ahmad and co-workers (2003) extracellularly synthesized monodispersed and spherical gold nanoparticles (AuNPs) of 8 nm (average size) using the actinomycete, *Thermomonospora* sp. Kowshik *et al.* (2002) produced extracellular silver nanoparticles (Ag-NPs) by a silver tolerant strain of yeast MKY3, which synthesized 2 – 5 nm hexagonal Ag-NPs in a log phase of growth. Predominantly, fungi are considered to be the extracellular nanoparticle producing microorganisms because they possess tremendous secretory

elements that plays a role during the reduction and capping of nanoparticles (Narayanan and Sakthivel, 2010). In another study, it was observed that *Colletotrichum* sp., an endophytic fungus isolated from the leaves of geranium plant (*Pelargonium graveolens*) rapidly reduces gold ions to zero-valent AuNPs (Shankar *et al.*, 2003).

Bacteria are able to synthesize nanoparticles intracellularly or extracellularly with good morphology. In the intracellular technique, ions are transported into the cell for nanoparticle formation in the presence of enzymes (Li *et al.*, 2011), while in the extracellular method, metal ions are trapped on the surface of the cells and reduced in the presence of enzymes (Zhang *et al.*, 2011). Extracellular production of metal nanoparticles take place when the reduction process of the metal ion involves the cell wall reductive or soluble secreted enzymes (Narayanan and Sakthivel, 2010). Industrial applications favour extracellular nanoparticles compared to those produced intracellularly. This may be due to the supplementary processes (ultrasound treatment or reaction with suitable detergents) required for intracellular nanoparticles release (Narayanan and Sakthivel, 2010). Considerably, bacteria may become biofactories for synthesis of nanoparticles such as gold, silver, platinum, titanium, cadmium sulphite etc. (Iravani, 2014).

Many studies have investigated Ag-NPs widely because they possess great chemical, physical as well as biological characteristics including inherent superiority which stems mainly from the size, shape, composition, crystallinity and structure of Ag-NPs compared to their bulk form (Sun and Xia, 2002; Kumar *et al.*, 2008; Atwater and Polman, 2010; Desireddy *et al.*, 2013; Syafiuddin *et al.*, 2017). These particles also possess a high electrical and chemical stability, surface-enhanced Raman scattering (SERS) and non-linear optical behaviour (Krutyakov *et al.*, 2008). Similar to their bulk counterpart, Ag-NPs are effective antimicrobial agents against Gram-negative and Gram-positive bacteria (Durán *et al.*, 2005), including extremely multi-resistant strains such as methicillin resistant *Staphylococcus aureus* (MRSA) (Nanda and Saravanan, 2009).

Silver has been used widely in preparation of various antimicrobial agents for the past few years because it possesses antimicrobial activity (Klapiszewski *et al.*, 2015; Xia *et al*., 2016) Today it is used to produce Ag-NPs for different applications in the fields of medicine, food, health care, etc. (Huang *et al.*, 2019). Effects of silver ions and Ag-NPs have been shown to be bacteriostatic and/or bactericidal against strains such as *E. coli*, *S. aureus* including yeast (Keat *et al.*, 2015). However, Méndez-Vilas (2011) reported that complexes formation for silver ions is limited while the silver ions effect remain briefly. Nonetheless, Mohammed (2015), indicated that the application of Ag-NPs which possess novel antibacterial effects by generating reactive oxygen species (ROS) such as hydrogen peroxide has resolved this drawback. Studies have displayed the antimicrobial activities of Ag-NPs to be highly effective on

Gram-negative bacteria (including highly resistant bacteria) in contrast to Gram-positive bacteria (Shrivastava *et al.*, 2007; Singh *et al.*, 2008).

Some of the antimicrobial activities of Ag-NPs are listed here considering their biocidal effectiveness against a large number of microorganisms. The antifungal effects of Ag-NPs on *Candida albicans* involves disrupting the cell membrane structure leading to inhibition of reproduction (Kim *et al.*, 2009). Wan *et al.* (2019), studied the Ag-NPs effect on Kaposi's sarcoma-associated herpesvirus (KSHV) and Epstein-Barr Virus (EBV)-associated tumour cells *in vitro* and *in vivo*. They discovered that for the first time Ag-NPs presented exclusively higher cytotoxicity on KSHV/EBV-latently infected cells through reactivating viral lytic replication, and that further blocking KSHV primary infection via direct termination of the virion particles. Silver nanoparticles could serve as an alternative treatment of infectious disease due to the rising antibiotic and antimicrobial resistance (AMR) by disease-causing organisms.

Antibiotic discovery and their development in diseases treatment is regarded as the greatest achievement in the history of drug development (Peterson and Kaur, 2018). However, antibacterial drugs have been overused globally over the past decades and the misuse of these drugs in both humans and food-producing animals have led to the increase in bacterial resistance (WHO, 2014). The development and extensive use of antibiotics has helped prolong the lives of billions of people. However, the frequent use of antibiotics presents accelerated development of resistance by bacteria to these antibiotics, making treatment of infections a much more difficult task (Pramanik, 2016). The total global consumption of antibiotics was reported to have increase by more than 30%, roughly from 50 billion to 70 billion standard units (SU) between 2000 and 2010 (Pramanik, 2016). Naveed *et al.*, (2015) reported that the misuse of antibiotics commonly occurs in cases of diarrheal illnesses and respiratory diseases. This practice is common in developing countries (Tzialla *et al.*, 2012) where antibiotics are purchased without a prescription (Naveed *et al.*, 2015). Antimicrobial resistance has become a major problem in both medical and public health due to its direct association with disease management (Ramamurthy, 2008).

The increase in antibiotic resistance particularly in hospitals has been noticed since early 1960s and currently regarded as a critical threat to health care with high mortality rates including health care costs (Pramanik, 2016). Reduced efficacy of several disease treatment drugs including antibacterial and antiviral drugs due to AMR results in difficulties in disease treatment, high cost, or even impossible to treat diseases. The impact of which is most obvious in particularly vulnerable patients, leading to prolonged illness and higher mortality rates (WHO, 2014). The United States (US) health care has been reported to exhaust approximately $21 – $34 billion annually, along with more than 8 million extra days in hospital (WHO, 2014). The European Centre for Disease Prevention and Control (ECDC) has estimated nearly 1.5 billion euros per year during the

last decade for direct infections cost arising from some of the most important antimicrobial resistant bacteria in the European Union (EU), Iceland and Norway (ECDC/EMEA, 2009). There is an excessive use of antibiotics in high-income countries and therefore they should reduce antibiotic consumption for both humans and animals (Antoñanzas and Goossens, 2019).

One of the present critical risk factors is that clinically important bacteria are not characterized by resistance to a single antibiotic only but by resistance to multiple antibiotics too (Carey and Cryan, 2003). The development of multi-drug resistant (MDR) and extremely-drug resistant (XDR) strains is a critical concern, resulting in few options to treat such resistance carrying infectious pathogens (Peterson and Kaur, 2018). These strains are superbugs arising from common human bacteria, having developed resistance and therefore increased virulence. They include MRSA and vancomycin resistant enterococci (VRE) strains, or opportunistic environmental bacteria which are intrinsically resistant such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Wright, 2007; Miller *et al.*, 2014).

Peterson and Kaur (2018) stated that it is natural for microorganisms producing antibiotics to also produce defence mechanisms for protection against their own antibiotics. Furthermore, it is believed that cohabitation of antibiotic-producing and non-producing bacteria is among the causes of co-evolution of resistance mechanisms in non-antibiotic-producing environment. There are other organisms which do not produce antibiotics, yet they carry degradation enzymes and vice versa (Peterson and Kaur, 2018). These are some of the factors that lead to the increasing spread of antibiotic resistance by most species which is a concern leading to the need for new methods to treat antimicrobial resistant strains.

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1.2 Problem statement

Many water pathogens are becoming drug-resistant due to the misuse of antibiotics and accelerated development of self-resistance mechanisms. Antibiotic resistant bacteria and their genes have been detected in several water sources including tap and bottled water (Wang *et al.*, 2016), untreated wells (Zhang, H. *et al.*, 2015; Zhang, T. *et al.*, 2015) as well as rivers and lakes (Jiang *et al.*, 2013). Antibiotic presence in rivers and lakes which results in antibiotic resistance development, complicates management of these water systems (Schwartz *et al.*, 2003). This is because the systems are primary water sources used widely for drinking water (Xi *et al.*, 2009), irrigation and recreational purposes (Liu *et al.*, 2018). Processes used to treat drinking water are frequently not formulated to eliminate antibiotic resistance (Sanganyado and Gwenzi, 2019). Furthermore, they may even aid the emergence, distribution and channelling of antibiotic resistance bacteria from the environment to humans through horizontal gene transfer of antibiotic resistant genes.

The deterioration of water quality and development of antibiotic resistant bacteria and presence of their genes in African rivers results from large disposal of urban wastewater effluents into the rivers (Sibanda *et al.*, 2015). Several technologies such as membrane filtration, advanced oxidation processes and nanotechnology were established and currently being used to treat drinking water (Sanganyado and Gwenzi, 2019). Silver nanoparticles could present a favourable alternative to treat several common bacterial infections. The present study anticipates that Ag-NPs produced by *Enterobacter xiangfangensis* Pb204 will exhibit antimicrobial effects against common water pathogens namely, *Enterococcus faecium, S. aureus, A. baumannii, Klebsiella pneumoniae, P. aeruginosa, E. cloacae, E. coli* and *Vibrio cholerae* at a dosage lower than that commonly used in antibiotics.

The bacteria *E. xiangfangensis* was isolated from acid mine decant produced by a uranium mine in the West Rand, Gauteng (26°06'26.8"S 27°43'20.2"E). It was identified as an isolate of *Enterobacter* sp. by 16S rRNA and was assigned to identity *Enterobacter* sp. Pb204, then later *Enterobacter xiangfangensis* (Ho *et al.*, 2018). A study by Vallabh (2016) demonstrated resistance of the bacterium to lead (Pb), cobalt (Co), manganese (Mn), zinc (Zn) and nickel (Ni). Furthermore, synthesis of Ag-NPs by *E. xiangfangensis* was reported (Hiebner, 2016). The presence of an integrative and conjugated elements (ICEs) containing genes coding for proteins involved in resistance to several heavy metals including silver was revealed by whole genome sequencing of the bacterium. The proteins involved in silver resistance include those in the complete *sil* operon: *silE, R, C, F, S, B* and *A* as well as *copG* (Ho *et al.*, 2018) further confirming the link between silver resistance and Ag-NP synthesis of the *Enterobacter* sp.

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1.3 Aim and objectives

1.3.1 Study aim

This study set out to compare the antimicrobial activity of biogenic silver nanoparticles to that of antibiotics used to treat common human pathogens that contaminate water and include the ESKAPE group of pathogens.

1.3.2 Objectives

To achieve this aim, the following objectives were outlined:

- To synthesize Ag-NPs using the cell free extract of *E. xiangfangensis* Pb204.
- To characterize Ag-NPs using transmission electron microscopy (TEM) in order to determine optimum reaction parameters for biosynthesis based on size, shape and distribution.
- To test the antimicrobial activity of the Ag-NPs and antibiotics against eight bacterial pathogens.

- To compare the Ag-NPs antimicrobial profile to that of antibiotics used in the present study.

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Chapter 2: Literature review

2.1 Emergence of antimicrobial resistance

Antibiotics are medicinal drugs used to kill or slow down infections caused by bacteria. The misuse of antibiotics has resulted in AMR to these drugs by microorganisms particularly bacteria. Antibiotic resistance occurs when bacteria are able to resist antibiotics effects. The emergence of AMR is a serious concern threatening public health, health care cost and mortality. More than a hundred thousand deaths are reported annually due to antibiotic resistance (Review on Antimicrobial resistance, 2014). The World Health Organization (WHO) recognizes antibiotic resistance as a major global threat due to its projected increase (WHO, 2014). The struggle against the emerging antibiotic resistance has transpired mainly in clinical, community and recently spreading to agricultural environment with an aim to alleviate transmission and prevent selection of resistant bacteria during antibiotic treatment (Bengtsson-Palme *et al.*, 2018). The inadequate knowledge on how and under which circumstances resistance development is facilitated by the environment, makes mitigation of emergence and spreading of mobile resistant factors problematic (Berendonk *et al.*, 2015).

Naturally, an organism that can produce antibiotics, should subsequently carry mechanisms for resistance against its own antibiotics (Peterson and Kaur, 2018). Bacteria that produce antibiotics have a variety of sophisticated mechanisms for self-resistance and very often they contain more than one mechanism at the same time, to ensure that they are completely protected from the biologically active modules they produce (Peterson and Kaur, 2018). Drug resistance mechanisms fall into a number of wide categories such as alteration/inactivation of drugs, drug binding site/target modification, cell permeability changes that reduces drug accumulation within the cell, including formation of biofilm (Li and Nikaido, 2004; Wright, 2005; Wilson, 2013). The main biochemical pathways for self-resistance mechanisms found in antibiotic producing organisms are highlighted in figure 2.1; for each a specific example is provided. A common method to render antibiotics ineffective is antibiotic modification in particularly where aminoglycoside antibiotics, chloramphenicol and β-lactams are involved (Peterson and Kaur, 2018).

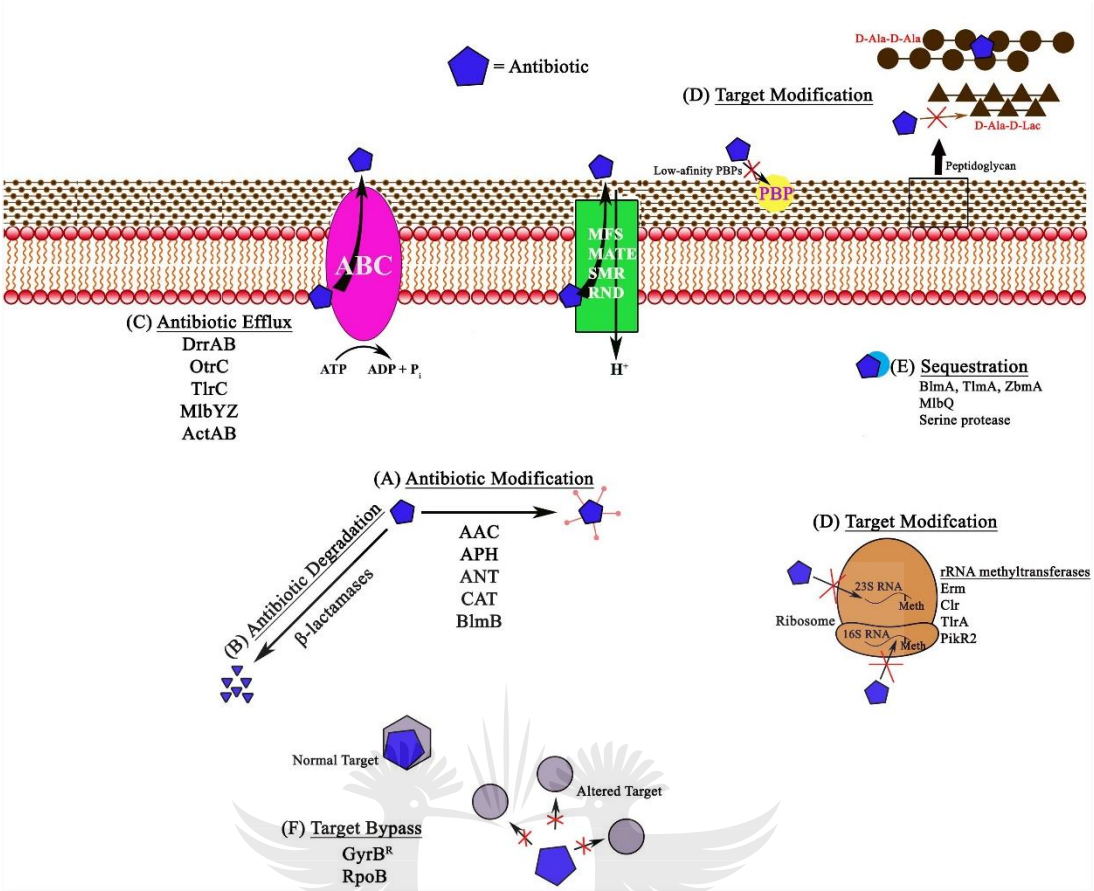

Figure 2.1. Illustration of different mechanisms of antibiotic resistance within bacteria presented with examples that include antibiotic modification (A), antibiotic degradation (B), efflux of antibiotics (C), target modification of antibiotics (D), antibiotic sequestration by special proteins (E) and antibiotic target bypass (F) (Peterson and Kaur, 2018).

There are species which do not generate antibiotics, however, they carry modification enzymes for resistance and vice versa. Streptomycin resistance is excluded from this because it has been established that antibiotic synthesis is correlated to the role of modification enzymes in self-resistance (Peterson and Kaur, 2018). Streptomycin 6-phosphotransferase is a modification enzyme that functions to convert streptomycin to inactive precursor streptomycin-6-phosphate in the producer *Streptomyces griseus* (Peterson and Kaur, 2018).

Antibiotic efflux usually works in combination with other resistance mechanisms such as antibiotic modification of the target. *Streptomyces peucetius* is an example of an antibiotic producer involved in antibiotic efflux. The organism produces daunorubicin (Dnr) and doxorubicin (Dox) anticancer antibiotics which are closely related and the two intercalate with DNA to avert further replication rounds (Peterson and Kaur, 2018). Efflux of these antibiotics in *S. peucetius* occurs by an ATP Binding Cassette (ABC) family transporter DrrAB coded by *drr*AB genes embedded within the genes responsible for biosynthesizing these antibiotics (Guilfoile and Hutchinson, 1991).

Sequestration involves drug-binding proteins which supresses antibiotics from reaching their target. The metal bound or metal-free antibiotic sequestration is involved in the primary resistance mechanism in the bleomycin family of antibiotic producers (Sugiyama and Kumagai, 2002). One or more genes of each of the bleomycin family producer is associated with ABC transporters in their biosynthesis cluster (Du *et al.*, 2000; Tao *et al.*, 2007; Galm *et al.*, 2009), capable of being applied for removal of the antibiotics bound to binding proteins.

Target modification functions as a mechanism of self-resistance against various antibiotic classes such as aminoglycosides, β-lactams, macrolides, lincosamides and streptogramins (MLS) (Peterson and Kaur, 2018). Target modification utilizes 16S rRNA methyltransferases for resistance against aminoglycosides in which methylation occurs at A1408 or G1405 residues (Shakil *et al.*, 2007). The structure of β-lactam antibiotic enables the association and acylation of the active site serine by the antibiotic which results in its inhibition (Yeats *et al.*, 2002).

Prevalent and haphazard antibiotics use since the discovery of antibiotics gave rise to resistant strains for all antibiotics that have been discovered to date. These observations anticipate that there may soon be development of resistant strains from all antibiotic used or in the long run. The spontaneous mutations rate also contributes to the emerging antibiotic resistance and extensively continuous exchange of DNA mechanisms in bacteria (Peterson and Kaur, 2018). One of the growing concerns in public health is the ESKAPE pathogens that comprise a group of bacteria with rapidly increasing MDR. Antimicrobial resistance in these organisms are expected to increase before long due to continuous changes in resistance profiles, resulting in the death of potential pipeline therapeutic agents (Santajit and Indrawattana, 2016).

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2.2 Antibiotic resistance in ESKAPE pathogens

ESKAPE pathogens are among the world's leading cause of infections associated with health care (Santajit and Indrawattana, 2016), especially in critically ill and immunocompromised individuals (Zhen *et al.*, 2019). The ESKAPE acronym is derived from bacteria primarily responsible for most antibiotic resistance namely; *E. faecium, S. aureus, K. pneumoniae, A. baumannii, P. aeruginosa* and *Enterobacter species*, with the first two being classified as Gram-positive bacteria followed by four Gram-negative bacteria. These are among the most common bacterial species in health-care acquired infections, threatening human health and becoming more resistant to antibiotics commonly used (Zhen *et al.*, 2019). Consequently, these pathogens are associated with the rising morbidity and mortality rates, accelerated healthcare costs and uncertainties of diagnostics leading to further doubts in traditional medicine (Santajit and Indrawattana, 2016). The focus of attention towards ESKAPE organisms can help in tackling the wide challenge of antibiotic resistance, especially MDR (Zhen *et al.*, 2019). Table 2.1 gives an insight into

the resistance mechanisms that each ESKAPE pathogen uses to render antibiotics ineffective.

Table 2.1. Examples of ineffective antibiotics and the mechanisms of antibiotic resistance in ESKAPE pathogens.

Resistant strain	**Gram −/+**	**Antibiotic/s subjected to resistance**	**Mechanisms of resistance**	**Antibiotics used for treatment**	**References**
Enterococcus faecium	Gram +	Vancomycin Ampicillin Cephalosporins Tobramycin	Antibiotic target modification Antibiotic modification	Daptomycin Amikacin Netilmicin	Lee *et al.* (2019) Santajit and Indrawattana, (2016)
Staphylococcus aureus	Gram +	Vancomycin Penicillin Daptomycin Oxacillin	Antibiotic target modification Antibiotic modification	Teicoplanin Daptomycin Vancomycin	WHO, (2014) Shrestha *et al.* (2018)
Klebsiella pneumoniae	Gram −	Penicillin Ciprofloxacin Carbapenems Cephalosporins	Antibiotic modification/ degradation	Carbapenems Fluoroquinolones	Wyres and Holt, (2018) Santajit and Indrawattana, (2016)
Acinetobacter baumannii	Gram −	Carbapenems Colistin	Antibiotic degradation	Carbapenems	Peterson and Kaur, (2018) Tiwari *et al.* (2020)
Pseudomonas aeruginosa	Gram −	Fluoroquinolones Imipenem Carbapenem	Antibiotic efflux	Colistin Doripenem Ciprofloxacin	Pang *et al.* (2019) Santajit and Indrawattana, (2016)
Enterobacter species (*Escherichia coli*)	Gram −	Penicillin Fluoroquinolones 3rd generation cephalosporins	Antibiotic modification/ degradation	Carbapenems Quinolones	WHO, (2014) Peterson and Kaur, (2018)

Mutations and acquisition of mobile genetic elements are known to cause resistance development in *E.* coli (WHO, 2014). Fluoroquinolone resistance is mostly attributed to mutation while acquisition of mobile genetic elements has been the case for broad-spectrum penicillins and resistance to third-generation cephalosporins (WHO, 2014). Nosocomial infections by MDR extended-spectrum β-lactamase (ESBL)-producing *E. coli* pose a serious health risk in hospital settings (Mehrad *et al.*, 2015). Pathogenic *E. coli* is has the capacity to cause a number of humans infections including diarrhoea, peritonitis, urinary tract infections (UTIs), bacteremia and colitis (Makvana and Krivol, 2015). In a case of resistant strains, carbapenems are usually used for treatment because drugs such as third-generation cephalosporins are ineffective.

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The ability of *A. baumannii* to develop into an MDR strain has enabled it to become an increasing threat to hospital patients. A combination of mechanisms including expression of β-lactamases, alteration of cell membrane permeability and increased efflux pumps expression contributes to drug resistance in *A. baumannii* (Zarrilli *et al.*, 2013). The emergence of carbapenemase-producing *A. baumannii* strains carrying imipenem metallo-β-lactamases and oxacillinase serine β-lactamases has been reported recently (Santajit and Indrawattana, 2016). These strains demonstrated resistance to both colistin, and imipenem and the combination of resistance genes gives them the ability to quell the action of most traditional antibiotic compounds (Vila *et al.*, 2007; Boucher *et al.*, 2009).

S. aureus can cause a number of infections, most commonly infections of the skin, bone, soft tissue and blood stream, and it is the frequent cause of most infections in postoperative wounds (WHO, 2014). Resistance to almost all early antibiotic classes (penicillin, amoxicillin and ampicillin) in *S. aureus* is mediated almost exclusively by determinants acquired through horizontal DNA transfer (Pantosti *et al.*, 2007). Strains of *S. aureus* resistant to penicillinase stable antibacterial drug, which are referred to as MRSA have acquired a novel gene (*mecA*) that codes for a novel penicillin-binding protein (WHO, 2014). MRSA is a major AMR pathogen responsible for a number of diseases throughout the world, from mild skin infections to fatal diseases (Loncaric *et al.*, 2019).

K. pneumoniae is the species within which several new genes coding for AMR were discovered before spreading to other pathogens (Wyres and Holt, 2018). Resistance to several antibacterial drugs arise mainly from horizontal transfer of mobile genetic elements such as transposons or plasmids (WHO, 2014). Although, the contribution of this strain to the overall AMR challenge cannot be quantified, existing research indicates that it has a broader ecological range, considerably more varied DNA structure, greater diversity of AMR gene and a higher plasmid burden compared to other gram-negative bacteria (Wyres and Holt, 2018). Predominantly, *K. pneumoniae* is responsible for infections caused by carbapenem-resistant bacteria worldwide and there are no clinically effective treatments for many patients infected with these bacteria (WHO, 2014).

E. faecium is associated with vancomycin resistance. It can cause severe morbidity and mortality in immunocompromised hosts (Lee *et al.*, 2019). Hypermutable DNA of *E. faecium* attributes to the rapid adaptation to antimicrobials by the strain (Lee *et al.*, 2019). *E. faecium* strains are able to acquire and spread resistance genes swiftly via mobile genetic elements such as transposons and plasmids that are present among bacteria (Clewell, 1990). Many *E. faecium* have acquired high level resistance to β-lactam through modification of the penicillin-binding protein 5 (PBP5), resulting in decreased β-lactam affinity, increased tolerance to β-lactam and a combination of the two above modifications which can exponentially increase resistance (Fontana *et al.*, 1984).

P. aeruginosa is a common pathogen in hospital intensive care units (ICUs) due to the propensity of its innate resistance to several antibiotics and antiseptics, and the ability to accumulate further resistance mechanisms to multiple classes of antibiotics (Pachori *et al.*, 2019). Drug resistance in *P. aeruginosa* arises from innate and acquired resistance mechanisms. Innate resistance involves presence of over expressed efflux pumps and low permeability of outer membrane (Santajit and Indrawattana, 2016), while acquired resistance comes about due to the acquisition of resistance genes or mutations in porins-encoded genes, penicillin-binding proteins and chromosomal β-lactamase (Oie *et al.*, 2009).

A major source of building up, spreading and amplifying drug resistance in organisms is ICUs, where there is a higher selection pressure for resistance development of drug-resistant pathogens (Esposito and Leone, 2007) because of high antibiotics use for infection treatment in patients (Pachori *et al.*, 2019). Furthermore, ICU patients are faced with a higher infection risk because they have immunocompromised system, use of multiple procedures, and invasive devices such as mechanical ventilation, central venous catheterizations (CVC) and urinary tract catheterizations (Ranjan *et al.*, 2014). The variety of resistance genes present in the environment indicates that there are a lot more resistance genes available for recruitment by pathogens (Bengtsson-Palme *et al.*, 2018). In 2017, WHO published a list of bacteria (*Pseudomonas* species and various Enterobacteriaceae including *Klebsiella* species and *E. coli*) for which there is an urgent need for new antibiotics to treat these pathogens regarded as of critical priority (Ortega-Huedo *et al.*, 2020). The report outlined a concern regarding resistance of fluoroquinolones and third generation cephalosporins (WHO, 2017).

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2.3 Economic burden emerging from AMR

The accelerating AMR affects not only mortality of humans and animals but also the cost used to take measures for prevention of diseases and spreading of resistance. The AMR economic cost can be described as the increasing cost of patient treatment with resistant infections relative to susceptible infections as well as the indirect productivity loss because of high mortality resulting from resistant infections (Shrestha *et al.*, 2018). One of the driving host factors of AMR is human consumption of antimicrobials, implicating different drug classes and propagating resistance in different pathogens (Shrestha *et al.*, 2018), which in turn affects the cost needed to fight resistance. Coast *et al*. (1996), argued that the omission of antimicrobial costs in economical evaluation was outlined partially by its quantification difficulties, with substantial uncertainties related to resistance mechanisms, lack and poor relevant data quality, and other methodological challenges (Coast *et al.*, 1998; McGowan, 2001). Although some studies published have analysed the antibiotic resistance burden on the economy, it can be concluded that the cost impact of antibiotic resistance on

the health sector, patients and society at large has not been measured adequately (WHO, 2014).

Shrestha *et al*. (2018), evaluated the economic cost of AMR of five ESKAPE pathogens excluding *E. faecium* per antibiotic used mainly in Thailand and the US. The overall economic AMR cost in the five pathogens due to drug resistance was $0.5 billion and $2.8 billion in Thailand and the US respectively. The antibiotic cost per SU between the two countries was different for a number of reasons. Thailand has a higher AMR burden amounting to 28 deaths connected to AMR per 100,000 relative to the US with 4.6 per 100,000. In addition, epidemiological profiles of the two countries are different. For instance, Thailand is associated with high burden of mortality that arise from *Acinetobacter* relative to MRSA dominance in the US. In South Africa an estimated 450,000 new cases of tuberculosis (TB) were reported in 2014 with 1.8% of the new cases and 6.7% of the previously treated cases estimated to have MDR-TB (National Department of Health (NDoH), 2015). The global health expenditure database of WHO (2016), suggests a lack of resources and infrastructure in middle- and lower-middle-income countries, making it difficult to evaluate the economic impact of AMR in those countries.

Most resistance cases are associated with longer hospitalization periods, expensive drug use, increased doctor visits and disabilities (Abushaheen *et al.*, 2020). The costs per patient total over $20 billion per year in the US health care costs (Golka *et al.*, 2014). Two common hospital-acquired infections (pneumonia and sepsis) was responsible for almost 50,000 deaths in America and cost the US health care system over $8 billion in 2006 (Eber *et al.*, 2010). The direct and indirect cost of AMR due to MRSA in Thailand was estimated at $29 million and $151 million, respectively (Shrestha *et al.*, 2018). Approximately $55 billion in the US is exhausted on antibiotic resistance, where health care costs and lost productivity per year account for $20 billion and $35 billion, respectively (CDC, 2013).

A substantial quantity of antibiotics is used in South African livestock, including numerous antibiotics that have been banned for use in other countries (Eagar *et al.*, 2012; Moyane *et al.*, 2013). Antimicrobial use in livestock is estimated to increase by 67% in 2030 and nearly double in Brazil, Russia, India, China and South Africa (Van Boeckel *et al.*, 2014). If AMR is unchecked, its high costs on the population and economy may result in medical poverty trap (Ahmad and Khan, 2019). The risk of medical poverty trap is mainly associated with low-resource areas, exposing vulnerable communities to a generational poverty which will increase deaths rate (Ahmad and Khan, 2019). There is an urgent need for more research on antimicrobial stewardship including other areas of infection prevention besides pure antibiotics medication (Huebner *et al.*, 2019).

Davey *et al*. (2013) described antimicrobial stewardship as co-ordinated efforts to promote the appropriate use of antibiotics to improve patients' outcome,

reduction of microbial resistance as well as decreasing the spread of multi-drug resistant organisms and unnecessary costs. The NDoH in South Africa advocated by South African Antibiotic Stewardship Programme (SAASP), developed an AMR National Strategy Framework (2014-2024) in response to the global strategy of WHO for combating AMR (NDoH, 2014; Mendelson, 2015). The strategy outlines measures to control AMR, reduce further increases in resistant microbial infections and improve patients' outcomes (NDoH, 2014). However, such strategies are not sufficient alone since microbial resistance has been shown to evolve and increase whenever new antibiotics are introduced. Further alternatives should be explored such as Ag-NPs which are effective against numerous microbial agents. Silver nanoparticles are considered as one of the effective antimicrobials in various microorganisms including resistant pathogens, due to their greater properties (Frattini *et al.*, 2005; Krutyakov *et al.*, 2008).

2.4 Silver nanoparticles and their properties

StatNano (2016) reported about 3509 nanotechnology products in many sectors including automotive, cosmetics, petroleum, sports and fitness, textiles, water and wastewater. Currently Nanotechnology Products Database (NPD, https://product.statnano.com) report about 8867 nanotechnology products where silver accounts for 942 products. This figure have tripled the number reported by Project on Emerging Nanotechnologies (PEN, http://www.nanotechproject.org) in 2013 where nanosilver accounted for 313 products. This can be attributed to competitive properties of Ag-NPs making them ideal for industrial application. Silver nanoparticles have distinguished physicochemical properties including a high electrical and thermal conductivity, chemical stability, SERS, non-linear optical activity and catalytic activity (Krutyakov *et al.*, 2008). The fraction of atoms present on the nanoparticles surface attributes to these properties which increases their thermodynamic stability (Roduner, 2006). The catalytic activity of Ag-NPs depends on their size, structure, shape, size distribution and chemical-physical environment (El-Nour *et al.*, 2010).

Physicochemical properties also have an effect on the dissolution of Ag-NPs and their biodurability (Utembe *et al.*, 2015). The shape of Ag-NPs may have an effect on the mechanisms of cellular uptake within the organisms resulting in modulation of cytotoxicity (Akter *et al.*, 2018). Aggregation and agglomeration properties are major factors in different rates of uptake and stability of nanoparticles in the cell (Cho *et al.*, 2011; Gliga *et al.*, 2014; Milić *et al.*, 2015). A number of different biological effects (oxidative stress, DNA damage, mitochondrial disfunction and permeabilization) across biological barriers are mediated by the size Ag-NPs (Riaz-Ahmed *et al.*, 2017).

2.5 Application of Ag-NPs in various industries

Silver nanoparticles are significantly interesting in modern nanotechnology research because they offer unique properties. They can be incorporated in

various applications such as catalysis, antiseptic agents (medical sector), food packaging, cosmetics, bioengineering and environmental applications (Keat *et al.*, 2015). Several accredited institutions including US Food and Drug Administration (US FDA), US Environmental Protection Agency (US EPA), Society of Industrial-Technology for Antimicrobial Articles (SIAA) of Japan, Korea's Testing and Research institute for Chemical Industry, and FITI Testing and Research Institute have approved products made with Ag-NPs (AZoNano, 2006). Keat *et al*. (2015) identified a wide range of areas (figure 2.2) where Ag-NPs can be applied.

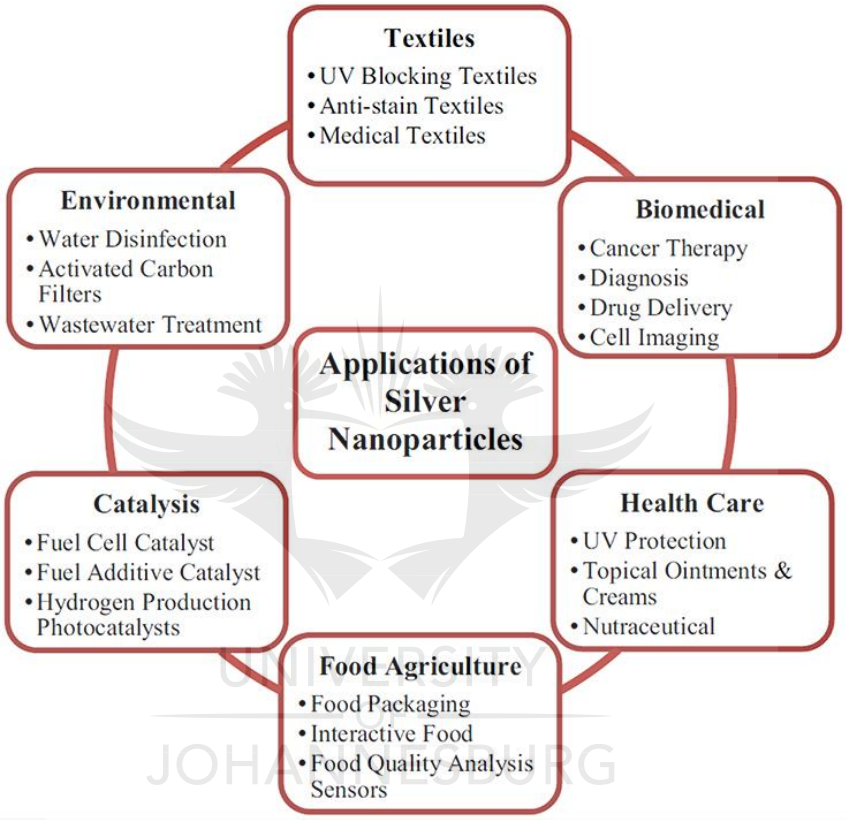

Figure 2.2. Applications of Ag-NPs in various industrial sectors ranging from health care, biomedical and textile industries to food, petroleum and water treatment. These include using Ag-NPs as antimicrobials in water treatment, antiseptic agents in food packaging and medical industry and catalyst fuel industry (Keat *et al.,* 2015).

In water disinfections, processes such as filtration, reverse osmosis, UV-treated filters, chlorination and advanced oxidation treatment, are commonly used for water treatment. However, they have drawbacks such as high cost (reverse osmosis) (Deshmukh *et al.*, 2018), limited activity (filtration) and conjugative transfer of antibiotic resistant bacteria (chlorination) (Sharma *et al.*, 2016). On the other hand, the use of Ag-NPs in water treatment has gained significant attention (Tran *et al.*, 2013). Water filters implanted with Ag-NPs have been shown to be efficient in removing and deactivating microbes through the use of two methods, metal disinfection and physical filtration (Deshmukh *et al.*, 2018). Silver nanoparticles are also being utilized in the food industry, such as the

development of smart materials for packaging food. Singh and Sahareen (2017) investigated low cost and environmentally friendly packets for vegetables storage which were embedded with Ag-NPs. After periodic determination, the shelf life of the vegetables was seen to increase while maintaining the nutrition value of the vegetables.

Silver nanoparticles are further used in medical application where they are impregnated in catheters, cardiovascular and bone implants to hinder biofilm formation and minimize pathogenic invasion risk (Tran *et al.*, 2013). Nano-crystalline dressing for wound therapy or hospital-acquired infections also uses Ag-NPs to minimize inflammatory response (Fong and Wood, 2006). Silver nanoparticles are preferable in textiles compared to traditional antimicrobial agents such as salts, quaternary ammonium compounds and triclosan (Deshmukh *et al.*, 2018), due to a number of factors including bactericidal resistance, low cost and environmental friendliness (Tamboli *et al.*, 2013; Wagener *et al.*, 2016). Generally, Ag-NPs can be considered as an ideal candidate for many commercial applications particularly in the biomedical industry (Keat *et al.*, 2015).

2.6 Antimicrobial activity of Ag-NPs on bacteria, fungi, yeasts and viruses

The antimicrobial effects of Ag-NPs are highly reported in various microorganisms including a range of bacteria, fungi, yeasts and virus, with high success rate of repression. The key mechanisms in antimicrobial activity of Ag-NPs can be simplified to their high surface area in releasing ions (Lee and Jun, 2019). Oxidization of Ag-NPs in aqueous environment takes place in the presence of oxygen and protons, releasing silver ions as the particle surface dissolves (Lee and Jun, 2019). However, the rate at which the ions are released is dependent on factors such as capping agent, colloidal state as well as of nanoparticle shape and size (Lee and Jun, 2019). For example, Sriram and colleagues (2012), demonstrated that small-sized or anisotropic Ag-NPs with a larger surface area was more toxic and released ions rapidly due to high surface energy emerging from highly curved or strained shape of nanoparticles.

Kim *et al.* (2007) reported suppression of growth on yeasts and minimum inhibitory concentration (MIC) between 6.6 and 13.2 nM. Pařil *et al.* (2017) studied the effects of Ag-NPs and copper nanospheres on wood-rotting fungi at a very low mass treatment of the Ag-NPs. Their study displayed highly efficient Ag-NPs activity against *Tinea versicolor* fungi as compared to *Poria placenta* fungi showing distinct antifungal effects of Ag-NPs on white and brown-rot fungi. Rajeshkumar *et al.* (2014) studied the antifungal activity of green synthesized Ag-NPs against *Fusarium* sp. Inhibition zones of 22.03 ± 0.033 mm in diameter were observed against the *Fusarium* sp.

Studies have demonstrated antiviral effects of Ag-NPs in different viruses such as human immunodeficiency virus (HIV) (Sun *et al.*, 2005; Lara *et al.*, 2010a, b), hepatitis B virus (Lu *et al.*, 2008), herpex simplex virus (HSV) (Baram-Pinto

et al., 2009, 2010) and influenza virus (Papp *et al.*, 2010). The specific Ag-NPs mechanism against specific viral infections may differ. In that regard, the potential mechanisms could rely on either interaction with surface glycoproteins of the virus, competition for virus binding to the host cells, viral particles inactivation before entry or impairing viral double-stranded DNA (Wan *et al.*, 2019). A study conducted by Lara *et al.* (2010a) to evaluate the antiviral activity of Ag-NPs on HIV-1 at non-cytotoxic concentrations revealed clear anti-HIV activity at an early viral replication stage. Silver nanoparticles of 10 nm have been reported to inhibit replication of hepatitis B virus (Lu *et al.*, 2008).

The exact mechanism of antimicrobial activity of nanosilver on bacteria is not fully understood but three toxicity mechanisms have been proposed. These include: (i) free silver ion uptake followed by disruption of ATP production and DNA replication, (ii) Ag-NPs and silver ions generation of ROS, and (iii) Ag-NPs direct damage to cell membranes (Marambio-Jones and Hoek, 2010). Silver nanoparticles have already been proven to exhibit toxic effects on both aerobic and anaerobic bacteria isolated from wastewater treatment (Choi and Hu, 2008). The strength of Ag-NPs effects is different with bacterial species (Lee and Jun, 2019) as they differ in structure, thickness and cell wall composition (Tamayo *et al.*, 2014). For instance, *E. coli* is more susceptible to silver ions compared to *S. aureus* due to differences in the thickness of their peptidoglycan layer in the cell wall (Lee and Jun, 2019).

Bera *et al.*, (2014) studied the size and shape dependent antimicrobial activity of Ag-NPs against *P. aeruginosa*, *S. epidermidis* and *B. megaterium* in which the smaller particles were able to penetrate the cell walls and enhance antimicrobial activity. In a separate study by Guzman *et al*. (2012), Ag-NPs proved to be effective against *E. coli*, *P. aeruginosa* and *S. aureus* at concentrations of 14.38, 6.74 and 14.38 ppm, respectively. Cavassin *et al.*, 2015) investigated the antimicrobial activity of Ag-NPs (citrate-stabilized, chitosan and polyvinyl alcohol PVA) against oxacillin-resistant *S. aureus*, VRE, carbapenem- and polymyxin B-resistant *A. baumannii*, carbapenem-resistant *P. aeruginosa* and carbapenem-resistant *Enterobacteriaceae*. Their study suggested that citrate and chitosan complexed Ag-NPs are best inhibitors against the strains tested. Nanoparticles exhibit great antibacterial effects especially in Gram-negative bacteria (Keat *et al.*, 2015). This is because of the bacterial cell wall structure which can easily be penetrated. Silver has thus been a driving force over the past decades to address the increasing threat of antibiotic resistance resulting from the misuse of antibiotics; leading to the development of different methods of Ag-NPs production for industrial application (Keat *et al.*, 2015).

2.7 Synthesis of Ag-NPs

2.7.1 Overview of nanoparticle synthesis

Metal nanoparticles are usually synthesized in either top-down or bottom-up technique. Bulk material are broken down to generate the required

nanomaterials in the top-down method, while single atoms and molecules are assembled into larger nanostructures to produce nano-sized materials when using bottom-up technique (Chugh *et al.*, 2018). Synthesis of Ag-NPs has been explored and conducted through various methodologies such as chemical, physical and more recently through biological approaches. There are advantages and disadvantages associated with each method, common problems being costs, scalability, particle size and distribution (Tran *et al.*, 2013). Furthermore, chemical and physical approaches tend to be more labour-intensive and hazardous, relative to biological methods which presents attractive properties such as high yield, solubility and stability (Zhang *et al.*, 2018). While nanoparticle synthesis through chemical and physical methods is more popular, the use of toxic chemicals significantly limits their biomedical application, particularly in clinical fields (Li *et al.*, 2011). Comparison between the chemical, physical and biological methods shows that biological methods are favourable because of economic efficiency, reproducibility, simple procedures and require less energy.

2.7.2 Chemical methods for Ag-NPs synthesis

Chemical synthesis of Ag-NPs can be subdivided into chemical reduction (Zhang *et al.*, 2011), irradiation-assisted chemical methods (Sotiriou and Pratsinis, 2010), electrochemical techniques (Roldán *et al.*, 2013) and pyrolysis (Sotiriou *et al.*, 2011). The chemical process usually employs three main components namely metal precursors, reducing agents and capping/stabilizing agents (Tran *et al.*, 2013). Reducing agents that are widely used include ascorbic acid, borohydride, alcohol, sodium citrate and hydrazine compounds (Wei *et al.*, 2015). Reducing and capping agents can easily be changed or modified to achieve desirable characteristics of Ag-NPs in terms of shape, size distribution and rate of dispersion (Pillai and Kamat, 2004). Distinct metallic salts are utilized to produce corresponding metal nanomaterials such as gold, silver, zinc oxide, copper, platinum, etc. (Kinnear *et al.*, 2017). During synthesis of nanoparticles, the strength and type of reducing agents and stabilizers should be considered to achieve nanoparticles of a specific size, shape with various optical properties.

2.7.3 Physical methods for Ag-NPs synthesis

Toxic chemicals are not employed in physical methods and processing is faster compared to chemical methods. However, high energy consumption is a major drawback in physical synthesis (Wei *et al.*, 2015). The methods usually include arc-discharge (Tien *et al.*, 2008), physical vapor condensation (El-Nour *et al.*, 2010), energy ball milling (Kosmala *et al.*, 2011) and direct current magnetron sputtering (Asanithi *et al.*, 2012). The evaporation condensation method is capable of synthesizing large quantities of high purity Ag-NPs without the use of chemicals that release toxic substance and jeopardize human health and the environment (Lee and Jun, 2019). Agglomeration is often a major drawback in the evaporation condensation method because capping agents are not used (Lee and Jun, 2019). Using the arc-discharge method, Tien *et al.* (2008) fabricated Ag-NPs suspension in deionized water without adding surfactants,

where silver wires were submerged in the deionized water and used as electrodes. Siegel *et al.* (2012) reported on the synthesis of AuNPs and Ag-NPs using direct metal sputtering into liquid medium.

2.7.4 Biological methods for Ag-NPs synthesis

Efforts have been made to utilize microorganisms as potential eco-friendly nanofactories for Ag-NPs synthesis (Mukherjee *et al.*, 2001; Ahmad *et al.*, 2003; Panáček *et al.*, 2006). Studies have reported that cell free extracts of some bacteria such as *Bacillus* sp., *E. coli*, *E. cloacae*, *S. aureus*, *K. pneumoniae*, *Lactobacillus acidophilus* and *P. aeruginosa* could induce Ag-NPs synthesis (Shahvredi *et al.*, 2007; Kalimuthu *et al.*, 2008; Saifuddin *et al.*, 2009). Toxic reducing agents and stabilizers in biological synthesis are replaced by nontoxic molecules such as proteins, carbohydrates and antioxidants (Wei *et al.*, 2015). These nontoxic molecules are usually produced by living organisms including bacteria, fungi, yeasts and plants. The potential mechanisms of biological synthesis include enzymatic reduction such as NADPH reductase and nonenzymatic reduction (Ge *et al.*, 2014). Biological agents tend to reduce metal ions quicker and at ambient temperature and pressure conditions (Tran *et al.*, 2013).

Findings from studies using bacteria to produce Ag-NPs indicate that the size and shape of nanoparticles formed varies between species. The reduction of Ag^{+} ions to Ag^{0} driven by electrons released from NADH in *Bacillus licheniformis* leading to formation of Ag-NPs was demonstrated by Lee and Jun (2019). In a separate study, Ag-NPs with a size range of 28.2 – 122 nm (average size of 52.5 nm) were produced when a culture supernatant of *K. pneumoniae* was incubated with $AgNO_3$ solution (Shahverdi *et al.*, 2007). Silver nanoparticle production also varies between different types of microorganisms. Fayaz *et al.* (2010) studied the use of *Trichoderma viride* for extracellular synthesis of Ag-NPs from $AgNO_3$ solution which resulted in Ag-NPs of 5 – 40 nm. Silver nanoparticles were synthesized by *Aspergillus terreus* resulting in spherical 8 – 20 nm particles (Balakumaran *et al.*, 2016). The nanoparticles exhibited antimicrobial effects against several bacteria including *K. pneumoniae*, *P. aeruginosa*, *S. aureus* and *E. coli*. Other parameters such as pH and temperature can influence the size, shape and distribution of biosynthesized Ag-NPs as is evident from these studies.

Synthesis of Ag-NPs using biological methods can take place within the cell or outside the cell. During the intracellular synthesis, the negatively charged cell wall interacts with the positively charged metal ions electrostatically and bioreduces the metal ions to nanoparticles (Thakkar *et al.*, 2010). In an extracellular synthesis, microorganisms are incubated with silver ions to generate Ag-NPs as result of intrinsic defence mechanism against the toxicity of the metal (Tran *et al.*, 2013). The mechanism of silver resistance with a focus on bacteria is further reviewed here.

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2.7.4.1 Silver resistance in bacteria

Silver resistance in bacteria has been reported and the resistance mechanisms that are encoded by various plasmid-based genes have been studied extensively (Silver, 1996; 1998; Silver and Phung, 1996). Microbial resistance to almost all toxic heavy metals arise from their chemical detoxification and energy-dependent ion efflux from the cell by membrane proteins that function either as ATPase or chemiosmotic cation or proton anti-transporters (Narayanan and Sakthivel, 2010). Microbial resistance to silver can also be affected by alteration in solubility. Therefore, metal ions can be detoxified by microbial systems using either reduction and/or precipitation of soluble toxic inorganic ions to insoluble non-toxic metal clusters (Narayanan and Sakthivel, 2010). Nies and Silver (1995) reported that the regulation of intracellular concentration of essential metal ions may result from sequestration or by changing the oxidation state of the metal ions (for some metals). However, the main regulation process for intracellular concentrations of inorganic cations and anions is by membrane transport systems.

The presence or absence of a metal ion transporter of certain specificity is dependent upon the metal ion, the bacterial species and the physiological state of the cell (Nies and Silver, 1995). The bulk metal (Ag^0) is relatively nontoxic because of poor bioavailability while the silver cation (Ag^+) is extremely toxic to most microorganisms (Muller, 2018). Bacteria are particularly susceptible to Ag^+ due to the extreme ability of the cation to pass through the membrane and adversely affect metabolic processes by nonspecific mechanisms that include binding to DNA, proteins, free thiol groups and interference with enzyme, electron transport, and membrane ion-exchange systems (Muller, 2018). The current international consensus is that there is relatively slight bacterial resistance to silver due to the nonspecific nature of Ag^+ toxicity (Chopra, 2007; Woods *et al.*, 2009; Elkrewi *et al.*, 2017), although the misuse of silver as an antimicrobial agent may lead to more resistance development by microorganisms in particular bacteria.

Certain bacterial species have developed the ability to resort to specific defence mechanisms to suppress stresses like toxicity of heavy metal ions or metals (Iravani, 2014). A study by Gupta *et al.* (1999), described the gene cluster responsible for silver resistance which contains a total of nine genes (*silP, ORF105, silA, silB, ORF96, silC, silSR* and *silE*). The *sil* genes have the following functions; *SilE* is responsible for a 143-amino-acid protein that binds to metal; *silSR* is responsible for a membrane sensor kinase and transcriptional regulatory responder; *silA* acts as an antiporter for inner membrane cation/proton; *silB* acts as a membrane fusion that binds outer and inner membrane of Gram negative bacteria together; *silC* functions as an outer membrane protein and *silP* is involved in the heavy metal resistance efflux P-type ATPase. The function of *sil* operon was elaborated further by a study conducted by Randall *et al.* (2015).

The study further demonstrated that in strains showing a phenotype of silver resistance, there is a prevalence of derepression of transporter expression owing to substitution of amino acid within the cognate sensor kinase of CusS or SilS. Despite the requirement of derepression of either CusCFBA or SilCFBA transporter for silver resistance, the increased efflux of silver that results is not sufficient on its own to achieve overt resistance phenotype (Randall *et al.*, 2015). This was further evident when the possibility of survival by Ag^+-sensitive microorganisms in Ag^+-rich environment was suggested to be far more common than currently believed due to achievement of silver resistance without the need for mutations or genetic material transfer between bacterial species (Muller and Merret, 2014; Muller, 2018). Consequently, relying on the presence of genetic markers (*sil* genes) as the only predictor of the extent to which microorganisms can survive silver, predominantly underestimates the precise survival capacity under non-laboratory conditions (Muller, 2018).

If AMR is unchecked, disease treatment will be ineffective affecting human and animal survival, agriculture, health care cost, GDP and subsequently accelerated mortality. Several countries have reported on the negative impact of AMR including the WHO, CDC, ECDC and NDoH, calling for interventions to combat and reduce the impact of AMR. These include the need for proper antibiotic prescriptions, quantification of antibiotic use per region, education on drug usage and alternative therapies. Nanoparticles particularly Ag-NPs have been identified as potential antimicrobials to alleviate AMR and possibly replace antibiotics. Biological Ag-NPs are used in various industries as described by Keat *et al.* (2015), including the highly sensitive biomedical sector. They are not only less costly than antibiotics but offer effective antimicrobial effects against various microorganisms attributed by their great properties, already described by several studies in this section.

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CHAPTER 3: Optimisation of reaction parameters for Ag-NP synthesis by *E. xiangfangensis* Pb204

Abstract

The ability of bacteria to reduce heavy metal ions to nanoparticles can be exploited in green chemistry. However, biogenesis often results in the production of nanoparticles that vary in size, shape and dispersity. This chapter focused on the biogenesis of Ag-NPs using a cell-free extract of *E. xiangfangensis* Pb204 and the optimization of synthesis parameters that would result in the production of Ag-NPs of uniform size and shape. An overnight culture of *E. xiangfangensis* Pb204 was added to 1 mM $AgNO_3$ for Ag-NPs synthesis. The reaction parameters (pH, temperature and time) were varied and the Ag-NPs produced ananalysed and characterised by TEM and EDX. Reaction pH affected nanoparticle distribution, shape and size while temperature and reaction time had effects mainly on size, nanoparticle quantity and reduction rate of metal ions into nanoparticles. Nanoparticles synthesized under pH 7, temperature of 37 °C and 24 hours reaction time were stable, small uniform-sized, spherical and well distributed. These parameters were maintained for all further syntheses of Ag-NPs in the study.

Keywords: Ag-NPs, *E. xiangfangensis* Pb204, biological synthesis, reaction parameters,

3.1 Introduction

Silver nanoparticles are attractive in various applications because they possess remarkable properties which include chemical stability, good conductivity, catalytic activity and antimicrobial activity. Silver nanoparticles of different sizes and shapes have different characteristics and they are highly toxic to numerous microorganisms (Katas *et al.*, 2018). Chemical and physical methods use hazardous chemicals during nanoparticles synthesis and tend to consume lots of energy (Sharma *et al.*, 2009). Chemically synthesized nanoparticles also have adverse effects from chemical residues when used in biomedical applications (Shankar *et al.*, 2004; Noruzi *et al.*, 2011). Biosynthesis of metal nanoparticles has gained interest because the method is eco-friendly and cost effectiveness (Wei and Qian, 2008).

The ability of bacteria to reduce heavy metal ions makes them an ideal candidates for nanoparticle synthesis. It appears that they can be used in synthesizing stable nanoparticles by optimizing reaction parameters and utilizing suitable bacteria resulting in nanoparticles with uniform size, good morphologies and compositions (Iravani, 2014). Nanoparticles synthesized extracellularly allow for size and shape control by adjusting the pH, temperature, substrate concentration and reaction time (Balakumaran *et al.*, 2016). *Bacillus* species have been used to synthesize Ag-NPs with various shapes (Vaidyanathan *et al.*, 2010) using naturally occurring reducing agents such as proteins and enzymes (Iravani, 2014). Silver nanoparticles with a well-

defined size were synthesized in aqueous $AgNO_3$ by *P. aeruginosa* (Klaus *et al.*, 1999). *Enterobacter xiangfangensis* Pb204 is among bacterial species able to synthesize metallic nanoparticles including Ag-NPs (Hiebner, 2016; Ho *et al.*, 2018). Integrative and Conjugated Elements of *E. xiangfangensis* Pb204 codes for 28 proteins that are involved in the resistance of several metals including Cu, Ag and Zn (Ho *et al.*, 2018). Furthermore, it is highly probable that the heavy metal resistance pathway encoded in the bacterium ICEs plays a role in metal uptake and ultimate reduction to nanoparticles. This chapter aimed to determine a set of reaction parameters that resulted in the biosynthesis of uniform-sized, small and spherical Ag-NPs by a cell free extract of *E. xiangfangensis* Pb204.

3.2 Methodology

3.2.1 Culturing of *E. xiangfangensis* Pb204

A glycerol stock of the bacterium *E. xiangfangensis* Pb204 previously isolated from an acid mine in the West Rand, Gauteng (26°06'26.8"S 27°43'20.2"E), was inoculated into Luria-Bertani (LB) broth (Sigma, USA) and incubated overnight at 37 ºC in a shaking (200 rpm) incubator (Labcon, USA) as a pre-inoculum. The pre-inoculum was further inoculated at a ratio of 1:100 (v/v) into LB broth for biomass accumulation. The culture was incubated for 24 hours at 37 ºC on a continuous shaking incubator (Labcon, USA). Thereafter, the cells were harvested by centrifuging at 5000 x g for 15 minutes at 25 ºC using a Multifuge XIR centrifuge (ThermoFischer, Germany). The cells were discarded while the supernatant identified as the cell free extract was used downstream.

The bacterial culture was propagated throughout the study using the same culture conditions as described above, by inoculating glycerol stock into LB broth. Freshly propagated cultures were maintained in glycerol stocks prepared by adding 700 µl of bacterial culture to 300 µl of sterile 100% glycerol (Sigma, USA). The stock cultures were maintained at -20 ºC until required for biomass accumulation.

3.2.2 Extracellular synthesis of Ag-NPs

The cell free extract of *E. xiangfangensis* Pb204 that was prepared as described above in section 3.2.1 was centrifuged (Multifuge XIR, ThermoFischer, Germany) again at 15000 x g for 15 minutes to pellet any remaining cell debris. The supernatant was added to 1 mM $AgNO_3$ (pH 7, Sigma, USA) in a 1:1 ratio and incubated (with continuous shaking) at 37 ºC for 72 hours with continuous observations for a colour change from pale yellow to brown indicating Ag-NPs formation. An experimental control consisting of the cell free extract and sterile distilled water (sdH_2O) in a 1:1 (v/v) ratio was treated under the same conditions. This was done in duplicates for both Ag-NP synthesis and their controls. The morphology, size and distribution of the Ag-NPs were analysed using TEM coupled with energy dispersive X-ray (EDX) spectroscopy as described in section 3.2.5 below.

3.2.3 pH dependent synthesis of Ag-NPs

The effect of pH on size, distribution and morphology of Ag-NPs synthesized by *E. xiangfangensis* Pb204 was investigated by synthesizing the Ag-NPs under varying pH conditions. The synthesis was done following the same procedure as described in section 3.2.2; however, the pH of the $AgNO_3$ was adjusted separately to pH 3, 5, and 9. Experimental controls as described previously were also included for each pH variable. Furthermore, the incubation period was reduced to 48 hours as Ag-NPs were determined to have been synthesized within this period. The resulting Ag-NPs were also characterized for size, morphology and distribution using TEM described in section 3.2.5.

3.2.4 Temperature dependent synthesis of Ag-NPs

The effect of temperature on size, distribution and morphology of *E. xiangfangensis* Pb204 synthesized Ag-NPs was also investigated by synthesizing the Ag-NPs under varying temperatures. This was performed at the optimum pH determined in section 3.2.3 The same procedure in section 3.2.2 was followed while the incubation temperature was varied at 25 ºC, 30 ºC and 37 ºC and included an experimental control for each variant. After determining the optimum temperature and pH, the reaction time was further reduced to 24 hours to determine if Ag-NPs could be synthesized within that period.

3.2.5 Characterization of Ag-NPs using TEM coupled with EDX

To characterize nanoparticles using TEM (JEM-2100, Jeol, Japan) operated at 20 kV, 10 µl of the sample was drop-cast on to a holey carbon-coated 200 mesh copper TEM grid (SPA, USA) and then dried overnight in a desiccator. Following desiccation, the sample was analyzed for size, shape and distribution at Spectrum (UJ). Image J Image Processing and analysis software (NIH, USA) was used to determine the size of the nanoparticles. EDX spectrum (JEM-2100, Jeol, Japan) was used for elemental analysis.

3.3 Results and discussion

Silver nanoparticles cytotoxicity has been used widely in food and healthcare industries (Sharma *et al.*, 2009). For instance, bandages coated with Ag-NPs can kill harmful microorganisms and allow better healing at the injured tissue (Lee and Jun, 2019). Furthermore, using Ag-NPs in food packaging prevents contamination allowing food to last long. One of the major factors that mediate numerous biological effects such as DNA damage, cellular uptake, and oxidative stress is Ag-NP size. The shape of Ag-NPs can also influence the level of particle toxicity and immunological effects in cells (Riaz-Ahmed *et al.* (2017). Aggregated Ag-NPs have lower surface area which limits their antimicrobial activity on microbial cells (Ahamed *et al.*, 2008), therefore well distributed nanoparticles will exhibit superior antimicrobial effects on microbial cells. It is therefore important to consider the reaction parameters used to synthesize Ag-NPs when using biological routes.

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When some microorganisms are incubated with metals, nanoparticles are produced as a result of detoxifying metal ions by either reduction or precipitation of soluble toxic inorganic ions to insoluble non-toxic metal nanoclusters (Narayanan and Sakthivel, 2010) such as Ag-NPs. Shkryl *et al.* (2018) synthesized stable Ag-NPs of 10 – 40 nm in size via a green synthesis method utilizing *LoSilA1*-expressing cali extract. Metal reducing *S. oneidensis* incubated with $AgNO_3$ produced stable, nearly monodispersed spherical Ag-NPs (2 – 11 nm size range) with an average size of 4 ± 1.5 nm (Suresh *et al.*, 2010). *Lactobacillus species* have also been reported to synthesize Ag-NPs using of $AgNO_3$ (Pugazhenthiran *et al.*, 2009). To achieve desirable nanoparticles with respect to morphology, size and dispersity, optimisation of reaction parameters such as pH, temperature, and Ag^+ concentration is important (Sintubin *et al.*, 2009; Suresh *et al.*, 2010; Amaladhas *et al.*, 2012; Umadevi *et al.*, 2012).

This chapter focused on the optimisation of reaction parameters, specifically pH, temperature and reaction time, in the synthesis of small, spherical and uniformly distributed Ag-NPs using the bacterium *E. xiangfangensis* Pb204. The synthesized Ag-NPs were intended for application as an antimicrobial treatment for ESKAPE pathogens as discussed in Chapter 4.

3.3.1 Visual confirmation of Ag-NP synthesis

Colour formation is the first step in the confirmation of nanoparticle formation. Biosynthesis of Ag-NPs was achieved by incubation of the cell free extract of *E. xiangfangensis* Pb204 with 1 mM $AgNO_3$ at a 1:1 ratio. A colour change from pale yellow to yellow-brown was observed after 24 hours, 48 hours and 72 hours incubation period. The colour change to light brown/brown occurs due to the reduction of silver ions in $AgNO_3$ solution (Poojary *et al.*, 2016), resulting as an excitation of surface plasmon vibrations caused by nanoparticles (Ghaffari-Moghaddam and Hadi-Dabanlou, 2014). The formation of colour change depends on incubation time (Rajeshkumar *et al.*, 2014), observed in slight colour differences per incubation period. In this study, the colour changed from pale yellow to yellow-brown increasing in intensity as incubation time was prolonged. This suggested a proportional relationship between the reaction time and colour intensity which correlates to an increase in concentration of Ag-NPs formed over time. Silver nanoparticles incubated for 72 hours had higher colour intensity compared to Ag-NPs incubated for 48 hours and 24 hours. As expected, no colour formation was observed for control reactions (culture supernatant + sdH_2O) due to the absence of $AgNO_3$ in which silver ions are usually reduced to metal nanoparticles.

3.3.2 Characterization of Ag-NPs using TEM

To determine the shape and morphology of the nanoparticles, TEM was used. TEM provides crucial information such as overall particle size, shape, distribution, etc. (Chaudhuri and Paria, 2012). TEM analysis were done for Ag-NPs produced by *E. xiangfangensis* Pb204 incubated at 37 ºC (pH 7) for 72

hours. The distribution of the nanoparticles as shown in figure 3.1 appears to be variable with the shape clearly observed as spherical. The Ag-NPs ranged in size from 4 – 36 nm Ag-NPs with an average of 16.7 ± 9 nm as determined by Image J analysis (figure 3.2).

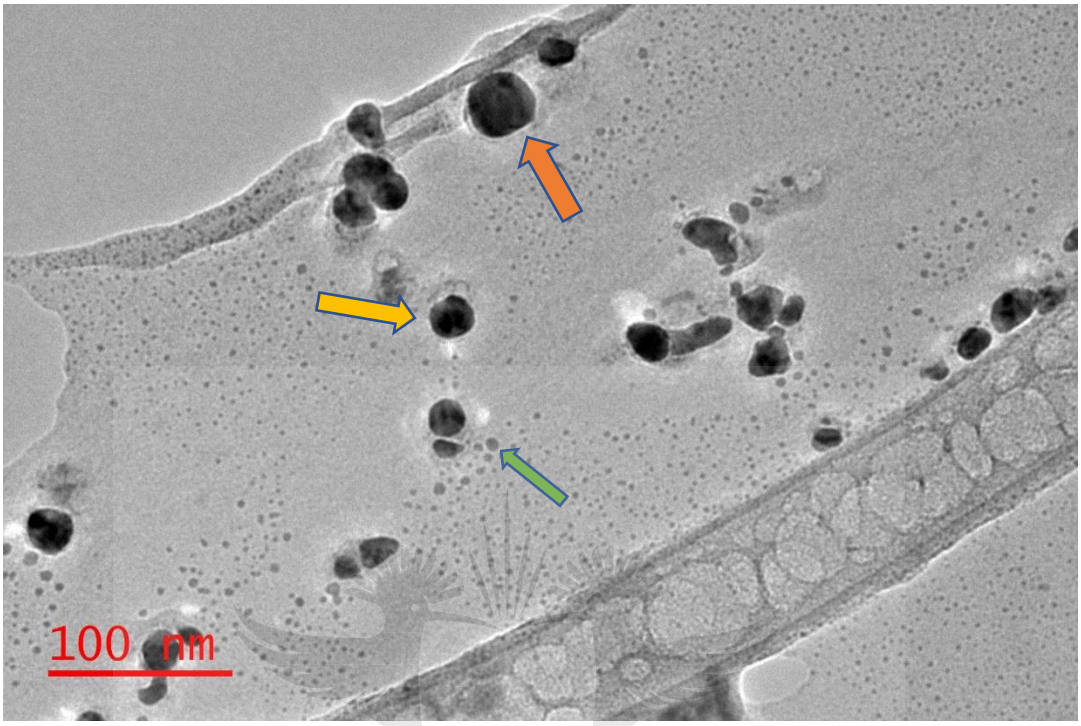

Figure 3.1. TEM image of Ag-NPs synthesized by *E. xiangfangensis* Pb204. The image shows spherical Ag-NPs of variable size and distribution formed when the cell free extract of *E. xiangfangensis* Pb204 was incubated with 1 mM $AgNO_3$ at 37 ºC for 72 hours. Arrows indicate Ag-NPs of different sizes; green displaying the smallest Ag-NPs produced and orange the larger ones.

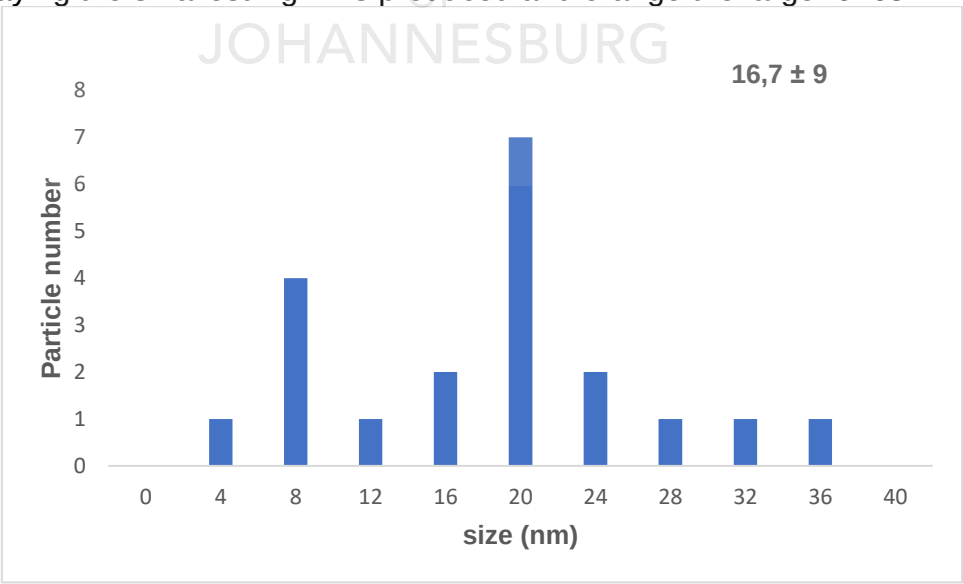

Figure 3.2. Histogram showing Ag-NPs size distribution when the cell free extract of *E. xiangfangensis* Pb204 is incubated with 1 mM $AgNO_3$ at 37 ºC for

72 hours. The average particle size of 16.7 ± 9 nm was determined using Image J Software analysis tool.

From figure 3.1, the majority of the Ag-NPs appear evenly dispersed with no aggregation. This is due to the stabilization of the nanoparticles by capping agents possibly being proteins secreted by the bacterium (Ahmad *et al.*, 2003; Saifuddin *et al.*, 2009). Uniform distribution is crucial in nanoparticle synthesis, since many nanoparticle have insufficient stability during preparations due to aggregation has somewhat delayed the development of commercial nanomaterial application (Saifuddin *et al.*, 2009). Image J Software (figure 3.2) was used to determine size distribution of the Ag-NPs. They were confirmed to range between 4 – 36 nm with a mean diameter of 16,7 ± 9 nm. Majority of the Ag-NPs were 20 nm in size followed by particles of 8 nm. These were not the desired Ag-NPs targeted in this study because they had a large size variation and were not evenly distributed with low yield which may affect their antimicrobial activity. Gliga *et al*. (2014) reported that variation in size influences the cytotoxicity of Ag-NPs. Several studies (Carlson *et al.*, 2008; Asharani *et al.* 2009; Yasin *et al.*, 2013) suggest that small Ag-NPs induce greater cytotoxicity, with enhanced penetration abilities against plasma membrane of the cells (Braydich-stolle *et al.*, 2010). It was therefore anticipated that with the optimisation of pH and temperature, Ag-NPs would be improved in terms of size and yield. The Ag-NPs were further characterised using EDX to confirm their elemental nature.

3.3.3 Characterization of Ag-NPs by EDX

Energy dispersive X-Ray spectroscopy is a tool used to characterise elements present in a sample. The technique characterizes materials in terms of their purity and stoichiometry, relying on a principle that unique atom structures will have unique X-ray emission spectrum (Khatua and Das, 2020). In figure 3.3 below, peaks are observed for carbon (C), silver (Ag) and copper (Cu) in different regions. The grids used to analyse the sample are made of Cu and coated with C which explains the presence of their peaks.

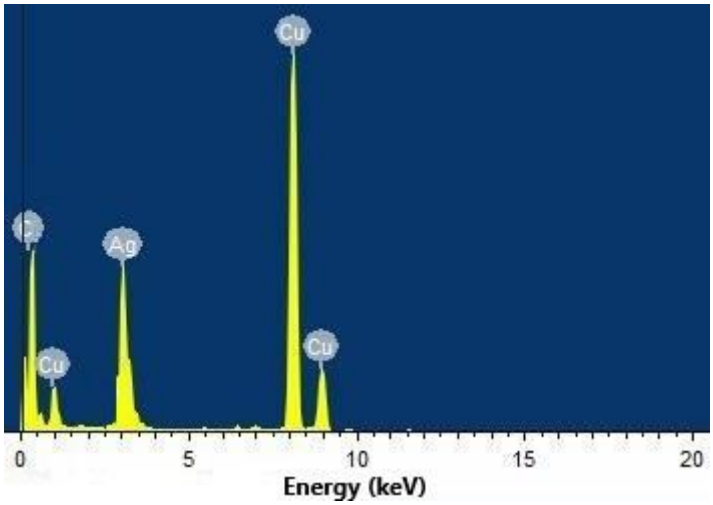

Figure 3.3. EDX image of Ag-NPs produced by *E. xiangfangensis* Pb204 showing an Ag peak at around 3 keV along with other elements (Cu and C) originating from the grids used during analysis.

Silver nanoparticle formation is confirmed by the presence of a peak in the silver region (Kalimuthu *et al.*, 2008). Analysis of the particles from the present study indicated a prominent Ag peak roughly around 3 keV confirming Ag-NPs synthesis (figure 3.3). A peak at approximately 3 keV is typical for Ag-NPs due to surface plasmon resonance (Magudapathy *et al.*, 2001).

3.3.4 Effect of pH on particle size, shape and distribution of Ag-NPs

Smaller Ag-NPs with definite shape and good distribution have been shown to exhibit greater antimicrobial activity compared to larger and agglomerated particles. Riaz-Ahmed *et al.* (2017) states that changes in Ag-NPs characteristics (size, shape, surface chemistry and aggregate propensity) have an effect on cellular uptake and cytotoxicity of the Ag-NPs. Furthermore, small Ag-NPs have been proven to possess superior toxicity, cell permeation and inflammatory effects over larger Ag-NPs. Reaction pH can significantly alter the electrical charges of biomolecules which could affect their capping and stabilizing agents and subsequently nanoparticle growth (Khalil *et al.*, 2014). Furthermore, in basic pH flocculation of Ag-NPs decreases significantly resulting in decreased aggregation of nanoparticles compared to acidic pH state. The effects of pH on the size of particles is dependent mainly on the reaction mechanisms involved. Hence a favourable pH is highly desirable to control the synthesis reaction (Chaudhuri and Paria, 2012) and subsequently yield particles suited to a particular application.

To determine if pH exerts any influence on the shape and size of Ag-NPs synthesized by *E. xiangfangensis* Pb204, a set of different reaction pH (3, 5, 7 and 9) were studied. The TEM images shown in figure 3.4 represent the effects that each pH condition had on the nanoparticles produced after synthesis at 37 ºC over 48 hours.

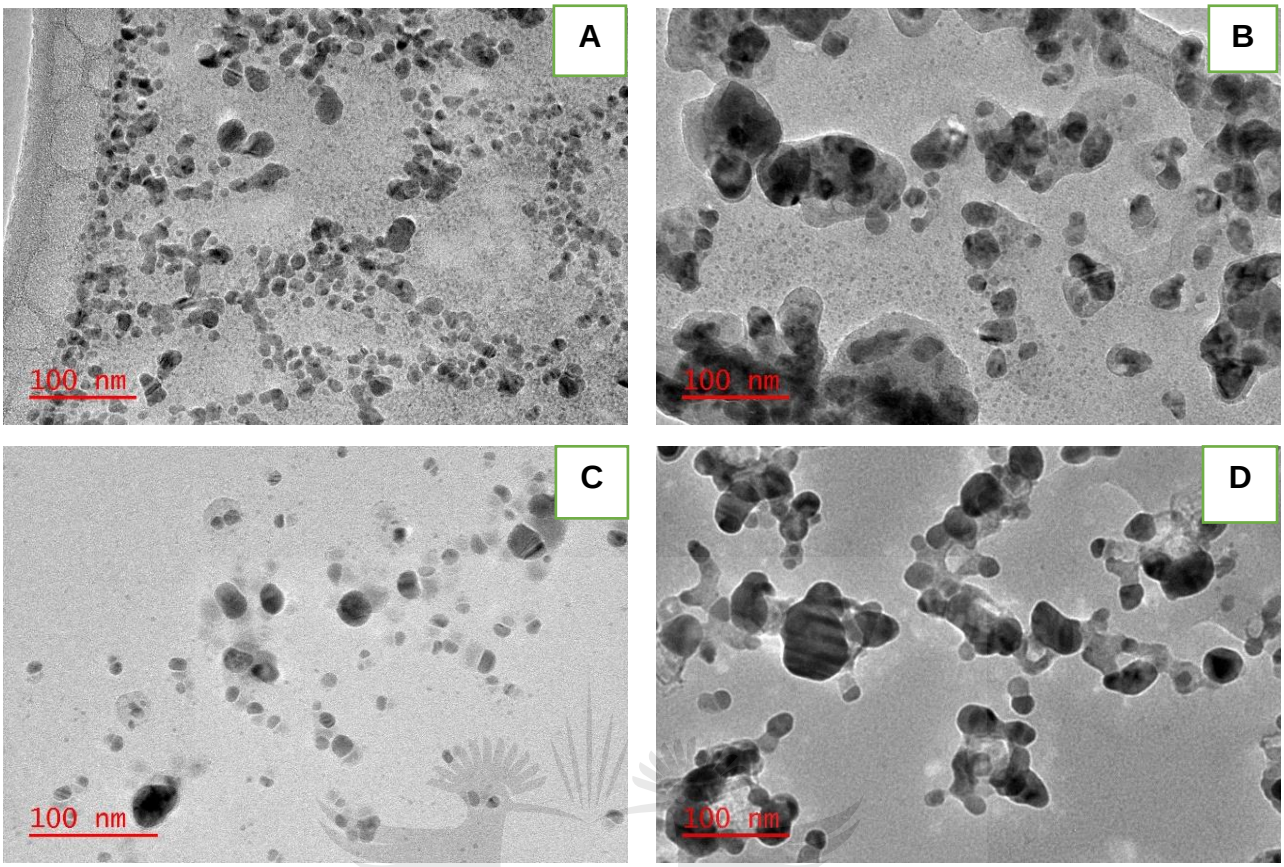

Figure 3. 4. Silver nanoparticles produced under different pH conditions when the cell free extract of *E. xiangfangensis* Pb204 was incubated with 1 mM $AgNO_3$ at 37 ºC for 48 hours. TEM images at 100 nm scale represent Ag-NPs synthesized at (A) pH 3, (B) pH 5, (C) pH 7 and (D) pH 9. Nanoparticles produced under acidic conditions had no definite shape with large variation in size. At a pH of 7, Ag-NPs appear well distributed with smaller size range and no agglomeration, while agglomeration of Ag-NPs was evident at an alkaline pH of 9.

From the TEM micrographs, agglomerated nanoparticles can clearly be seen in figures 3.4A (pH 3), B (pH 5) and D (pH 9) with the exception of figure 3.4C (pH 7) where little/no agglomeration is observed. In a study by Balakumaran *et al.* (2016), Ag-NPs produced by *Aspergillus terreus* at pH 3 and 4 were agglomerated while Ag-NPs formed at pH 7 were monodispersed and stable. The little agglomeration observed in figure 3.4C could be as a result of insufficient mixing of the sample before being drop cast onto the TEM grid and appears to be limited to the larger sized particles in the preparation. Vippola *et al*. (2009) stated that the agglomeration process might be affected by pH, electrolyte or salt content, and composition of proteins in the culture media. The high surface area of Ag-NPs also have an effect in their high agglomeration status in culture media (Bae *et al.*, 2013). Silver nanoparticles with high extent of agglomeration are less effective in inducing cytotoxicity effects in different cells compared to less agglomerated nanoparticles (Lankoff *et al*., 2012).

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Some previous studies (Chaudhuri and Paria, 2012; Khalil *et al.*, 2014; Poojary *et al.*, 2016) have reported that pH also plays a role in controlling shape and size during nanoparticle synthesis. This is also evident from figure 3.4 where the nanoparticles produced under the different pH conditions in the study varied in size. A definite trend in size was not obvious from the results but the smallest Ag-NPs were produced at a pH ≤ 7. Nanoparticles produced at pH 3 ranged between 6 nm and 34 nm in size (figure 3.4A) which was similar to that of 6 − 30 nm at a pH of 7 (figure 3.4C). However, a higher degree of particle agglomeration is observed at pH 3 as discussed earlier. A reaction pH of 5 yielded Ag-NPs with the largest variation in size from 6 nm to 61 nm. This correlates to the presence of large agglomerates visible in figure 3.4B. While the Ag-NPs synthesized at alkaline pH (9) were distributed over a smaller size range of 11 − 37 nm, the aggregation of these particles may affect their antimicrobial activity. In a study by Lok *et al.* (2006), Ag-NPs lost their antibacterial effect due to aggregation, however, antibacterial effect was restored by complexation of the nanoparticles with albumin which stabilized the Ag-NPs. Lankoff *et al.* (2012), studied the effects of aggregated Ag-NPs, revealing that highly aggregated nanoparticles are less effective on cellular level.

Several studies have investigated the effect of Ag-NP size on different cells and they have concluded that smaller particles can induce greater cytotoxicity (Elechiguerra *et al.*, 2005; Liu *et al.*, 2010; Lankoff *et al.*, 2012; Akter *et al.* 2018). This can be corroborated by a study in which Morones *et al.*, (2005) determined that only Ag-NPs of 1 − 10 nm had inhibitory effects on the growth of Gram-negative bacteria by disrupting cell membrane functions, penetrating into the cells and damaging the DNA. Most of the nanoparticles in this study appear to be spherical particularly those in figure 3.4 C and D, with some being asymmetrical (figure 3.4 A and B), which may affect their antimicrobial activity. This is because the cytotoxicity of Ag-NPs is influenced by factors such as size (Braydich-Stolle *et al.*, 2010; Gliga *et al.*, 2014), shape (Riaz-Ahmed *et al.* 2017; Akter *et al.*, 2018), aggregation (Lankoff *et al.*, 2012) and concentration of Ag^+ (Park *et al.*, 2010). However, there is insufficient data from studies to obtain a concrete idea on the complete cytotoxicity of Ag-NPs and their mechanism of toxicity (Akter *et al.*, 2018). After careful consideration, a reaction pH of 7 was used in subsequent syntheses due to the desirable nature (shape, size and distribution) of the nanoparticles produced under these conditions.

3.3.5 Effect of temperature and reaction time on particle size, shape and distribution of Ag-NPs

All syntheses of Ag-NPs using the cell free extract of *E. xiangfangensis* Pb204 in this study were previously performed at 37 ºC. The effects of temperature on the characteristics of Ag-NPs were investigated by further varying the reaction temperature at 25 ºC and 30 ºC while maintaining a reaction pH of 7 and reaction time of 48 hours. Only a few studies have reported on the effect of temperature on nanoparticles particularly in biological synthesis using bacteria.

This may be due to the ability to chemically synthesize nanoparticles in reactions conducted at temperatures ranging from room temperature (Chahar *et al.*, 2018) to around 100 ºC (Tran *et al.*, 2013) suggesting a similar versatility in biosynthesis. The size of nanoparticles has been reported to decrease as the temperature is increased (Fayaz *et al.*, 2010; Liu *et al.*, 2017). Khalil *et al.* (2014) synthesized small sized (20 – 25 nm) nanoparticles using olive leaf extract, where they increased the reaction temperature, leading to rapid reduction of Ag^+ ions and the subsequent homogeneous nucleation of silver nuclei.

In the current study, Ag-NPs produced at 37 ºC within a period of 48 hours had good morphology, small uniform size and spherical shape as seen in figure 3.4 C (section 3.3.4). When the temperature was set to 25 ºC, no particles were obtained indicating that the reaction did not proceed or did so very slowly over 48 hours resulting in too low a yield for visualisation by colour or TEM. The slow rate of Ag-NP synthesis at room temperature may be accelerated by increasing the reaction temperature (Khalil *et al.*, 2014). Figure 3.5 represents Ag-NPs synthesized at 30 ºC with continuous shaking over a 48 hour incubation period. The image shows low yield of Ag-NPs likely to be spherically shaped.

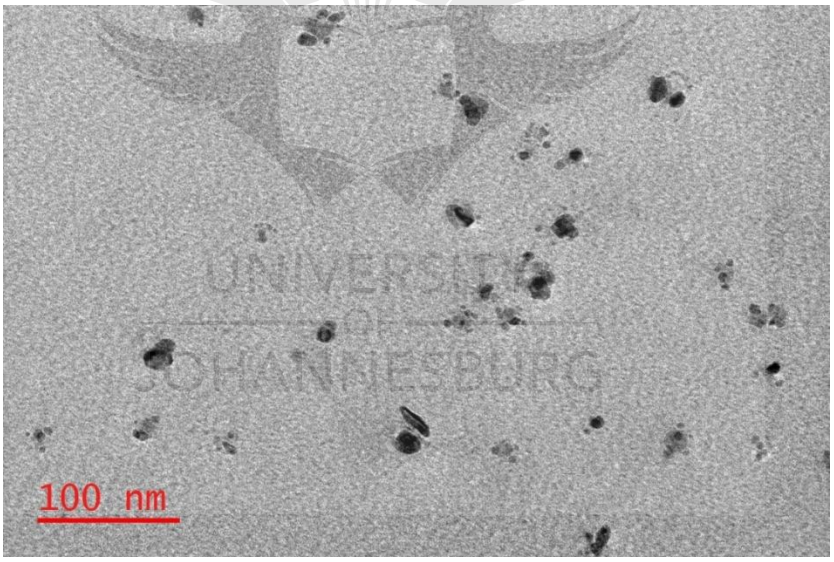

Figure 3. 5. TEM image of Ag-NPs produced using the cell free extract of *E. xiangfangensis* Pb204 in the presence of 1 mM $AgNO_3$ (pH 7) at 30 ºC for 48 hours. A low Ag-NP concentration was observed.

Increasing the reaction temperature from 25 ºC to 30 ºC did increase the rate at which Ag-NPs were made. This was evident from the visualisation of colour change and TEM results above (figure 3.5). An increase in the reaction temperature has been reported to result in the rapid biosynthesis of nanoparticles (Gurunathan *et al.*, 2009; Deepak *et al.*, 2011; Chaudhuri and Paria, 2012; Khalil *et al.*, 2014). Although, the low yield of Ag-NPs suggests that at 30 ºC, the rate at which Ag^+ ions are reduced is still slow. When compared to figure 3.4C (section 3.3.4), it can clearly be seen that allowing the

synthesis to proceed at 37 ºC for the same length of time increased the yield of Ag-NPs while maintaining their spherical shape. Based on these results, the reaction time under the same conditions (1 mM $AgNO_3$, pH 7, 37 ºC) was further reduced to 24 hours to evaluate how/if the nanoparticles would be affected. As shown in figure 3.6 differences in the Ag-NPs synthesized within 24 hours compared to 48 hours were observed.

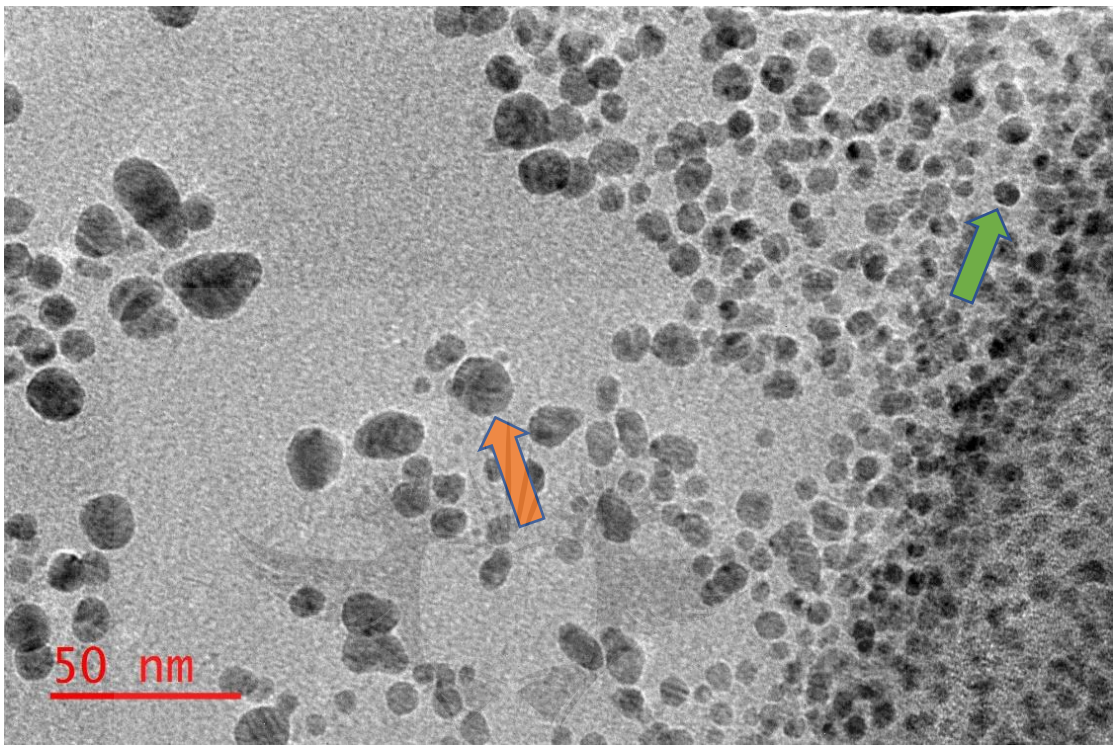

Figure 3. 6. TEM image of Ag-NPs produced using the cell free extract of *E. xiangfangensis* Pb204 in the presence of 1 mM $AgNO_3$ (pH 7) at 37 ºC for 24 hours. Uniform sized spherical Ag-NPs with a size range of 3 – 15 nm were observed.

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Comparing the results above with those synthesized at 48 hours under the same conditions, a longer reaction time resulted in an increase in particle size. Using Image J Software for measurement analysis, the Ag-NPs produced after 24 hours, ranged in size from 3 – 15 nm (average size of 7.80 ± 3.62 nm). Nanoparticles synthesized over 72 hours of reaction time had a wider size range of 4 – 36 nm (figure 3.1, section 3.3.2). This could be as a result of prolonged synthesis which enables the continued reduction of Ag^+ resulting in larger particles. Additionally, the uniform distribution of spherical Ag-NPs within this smaller size range can be observed in figure 3.6 suggesting that large differences in particle size can affect the homogenous distribution of the particles.

3.4 Conclusion

The study set to establish a set of reaction parameters for the production of homogenous, spherical Ag-NPs by *E. xiangfangensis* Pb204 through a process

of optimization. Biological synthesis of Ag-NPs has been investigated by many studies including the current one with an aim to establish uniform-sized, symmetrical shapes and good distribution of the nanoparticles. Silver nanoparticles are widely utilized in modern technology, however, there is still little understanding on their biological activity. Furthermore, several factors that include size, aggregation and agglomeration propensity, treatment duration plays a role in effectiveness and possibly safety of Ag-NP-coated medical devices, antimicrobial therapy and other application in which Ag-NPs are used. The optimum reaction parameters were established and determined to be a pH condition of 7, reaction temperature of 37 ºC and a reaction time of 24 hours, in this study, when *E. xiangfangensis* Pb204 is used to synthesis Ag-NPs. The Ag-NPs produced under these reaction parameters were small uniform-sized, spherical shaped particles with good distribution; characteristics which are essential for effective antimicrobial activity.

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Chapter 4: Comparing the antimicrobial activity of biosynthesized Ag-NPs with antibiotics used to treat ESKAPE pathogens.

Abstract

The ability of bacteria to acquire resistance genes has rendered most antibiotics ineffective. This may present Ag-NPs as a favourable alternative in fighting bacteria resistant to antibiotics. This chapter aimed to establish the antimicrobial activity of Ag-NPs against eight bacterial isolates that included the ESKAPE pathogens. The effect of Ag-NPs on *E. coli, P. aeruginosa, S. aureus, A. baumannii, E. faecium, K. pneumoniae, E. cloacae* and *V. cholerae* was determined by monitoring their growth spectrophotometrically in the presence of 0.1, 0.25, 0.5 and 1 mM as-prepared Ag-NPs. In addition, the activity of antibiotics used to treat these bacteria was tested using the Kirby-Bauer method where zones of inhibition were measured after 24 and 48 hours of incubation. Silver nanoparticles at lower concentrations of 0.1 and 0.25 mM did not exhibit any effect on all the bacterial strains tested. However, at 0.5 and 1.0 mM concentrations, antimicrobial activity against all strains was present and determined to be largely bacteriostatic. The dose of Ag-NPs required to inhibit bacterial growth was higher than that of the antibiotics commonly used to control these strains. Nonetheless, antibiotic activity is strain-specific while Ag-NPs were effective against all eight bacteria.

Keywords: Antibiotics, antimicrobial activity, ESKAPE pathogens, inhibition.

4.1 Introduction

Many common and yet lifesaving medical procedures rely on antibiotics to mitigate the risk of clinical infections (WHO, 2014). However, the misuse of antibiotics in clinical medicine has contributed to problems such as development of antibacterial resistance, increased health service costs, accelerating chronic disease burden and development of side effects (Alumran *et al.*, 2012). The bacterial capacity to acquire various antibiotic resistant genes continues to increase as more antibiotics are used worldwide (Abushaheen *et al.*, 2020). ESKAPE pathogens are a group of multidrug resistant bacteria classified as priority pathogens because they are the leading cause of life-threatening hospital acquired infections (Santajit and Indrawattana, 2016). Treating them has been a complex and challenging subject for at least a few decades. Consequently, many countries have had to adopt mitigation plans such as antimicrobial stewardships and actively seek alternative treatments.

The use of Ag-NPs to treat antibiotic resistant pathogens has been receiving much attention. Silver nanoparticles have been tested for their antimicrobial activities and proven effective in a number of studies (Choi and Hu, 2008; Guzman *et al.*, 2012; Pařil *et al.*, 2017; Jackson and Smith, 2018; Khan *et al.*, 2020). In this chapter, the antimicrobial activity of the as-synthesized Ag-NPs produced using the cell free extract of *E. xiangfangensis* Pb204 under optimised conditions (Chapter 3) was determined against ESKAPE pathogens.

Furthermore, the dosage of Ag-NPs required to inhibit the growth of the ESKAPE pathogens was compared to that of the specific antibiotic commonly used to treat each pathogen in the group.

4.2 Methodology

4.2.1 Propagation and maintenance of bacterial isolates

The bacterial culture was propagated using the same culture conditions as described in section 3.2.1 of chapter 3, by inoculating 50 µl of the glycerol stock into 4.95 ml LB broth. Then after incubation the culture were maintained in glycerol stocks prepared by adding 700 µl of bacterial culture to 300 µl of sterile 100% glycerol (Sigma, USA). The stock cultures were maintained in a freezer at -20 ºC temperature until required for biomass accumulation. The bacterial isolates (*E. coli, P. aeruginosa, S. aureus, A. baumannii, E. faecium, K. pneumoniae, E. cloacae* and *V. cholerae*) were obtained from the Water and Health Research Centre (University of Johannesburg, South Africa).

4.2.2 Antibiotic susceptibility test of the bacterial isolates

A total of six antibiotics, meropenem, ampicillin, cloxacillin, ciprofloxacin, vancomycin and combination-type penicillin (amoxicillin and clavulanic acid) (Mast Laboratories, UK), at different doses were tested for their activity on the eight pathogenic strains used in this study. Pure cultures of each bacterial strain were prepared as indicated in section 4.2.1 using the culture conditions described in section 3.2.1 and standardised by diluting to an OD_{600} of 1.00. One hundred microliters of a standardised strain was spread evenly on Mueller-Hinton agar (Sigma, USA) plates and discs impregnated with the specific test antibiotic were placed on the plates. This was done in triplicate for each antibiotic. The plates were then incubated at 35 ºC for 48 hours.

The zones of inhibition were measured with a ruler in mm and the triplicate measurements were averaged. Clinical Laboratory Standards Institute guiding document M100 Performance Standards for Antimicrobial Susceptibility Testing (27^{th} edition,2017) was used as a reference to determine whether the bacterial strains were susceptible, intermediate or resistant to the antibiotics where applicable.

4.2.3 Growth of bacterial pathogens in the presence of Ag-NPs

Silver nanoparticles were tested for their antimicrobial activity against the eight bacterial strains described in section 4.2.1 above. This was performed by assessing the growth of each strain in the presence of different concentrations of Ag-NPs using 96-well microplates (Thermo Fisher Scientific, UK). Overnight cultures of each of the above strains were diluted in LB Broth (Sigma, USA) to an OD_{600} of 1.00. A stock solution of 50 mM Ag-NPs was used to dilute the particles to the final desired concentration. Each designated well consisted of a total volume of 200 µl made up of 10 µl of a specific bacterium and Ag-NPs at a final concentration of either 0.1 mM, 0.25 mM, 0.5 mM or 1.0 mM diluted in LB broth (Sigma, USA). In addition to determining the antimicrobial effects of

the Ag-NPs, growth inhibition of each strain by 1 mM $AgNO_3$ was also evaluated. A growth control comprising only the bacterium in LB broth (Sigma, USA) was included for each strain. Each variable including the controls were tested in duplicate for all bacterial strains as shown in figure 4.1 below. The 96-well microplate was loaded into an xMark Microplate absorbance spectrophotometer (Bio-Rad, USA) for incubation.

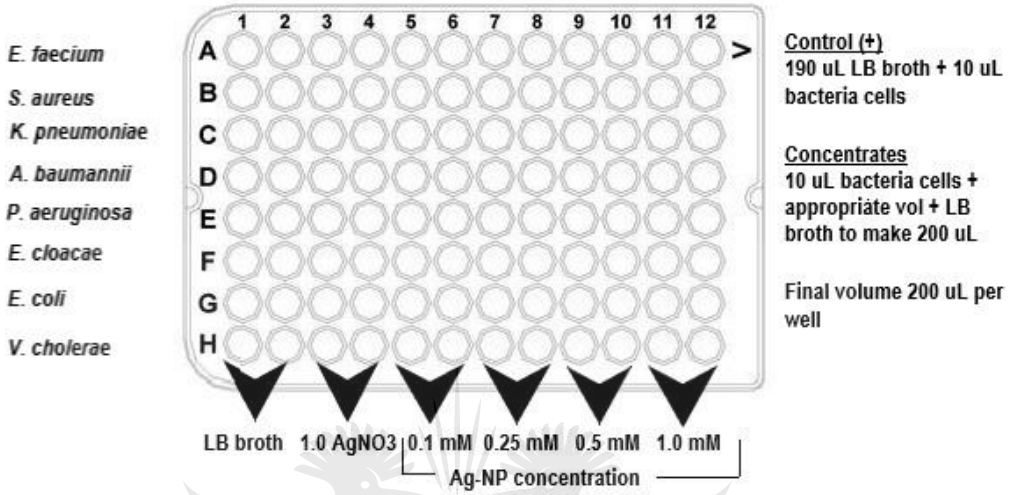

Figure 4.1. Experimental set up of the microplate used for antimicrobial assay of Ag-NPs and $AgNO_3$ against the eight bacterial pathogens over a period of 24 hours incubated in an xMark Microplate absorbance spectrophotometer.

With the use of Microplate Manager® 6 Software (Bio-Rad, USA), the xMark Microplate absorbance spectrophotometer was set to shake continuously using orbital mixing with medium speed at 35 ºC. Absorbance readings (duplicate) were taken over 24 hours with a 2 hour reading interval in a single optical density of 600 nm (OD_{600}) in kinetic mode and used to plot growth curves for each bacterial strain.

4.3 Results and discussion

ESKAPE pathogens are the leading cause of untreatable infections due to their widespread drug resistance. This prevalence of AMR can contribute to the emergence of a post-antibiotic era in which minor injuries and common infections becomes a leading cause of death (Alam *et al.*, 2019). Therefore, implementation of effective policies and programs through collaboration of multiple stakeholders is necessary to manage this crisis (WHO, 2016). The gap between new antimicrobial discovery and development of resistance highlights the need to focus on natural antimicrobial compounds (Varela and Kumar, 2019), for effective drug development. However, numerous compounds identified as potential antibacterials by *in vitro* studies are yet to reach clinical application owing to several drawbacks such as economic considerations and regulatory procedures involved in drug testing and approval for clinical use (Varela and Kumar, 2019). Antibacterial agents from nanomaterials have been

identified as potential replacements for traditional antibiotics as they continue to fail repeatedly (Zaidi *et al.*, 2017). Antimicrobial nanoparticles offer targeted therapy via specific accumulation, improved solubility, controlled drug release and broad therapeutic index (Huh and Kwon, 2011).

Nanoparticles, particularly Ag-NPs are the most widely studied due to their greater antimicrobial activities and ability to kill bacteria using different mechanisms (Zaidi *et al.*, 2017), making it difficult for microbes to develop resistance against them (Rai *et al.*, 2012). Furthermore, combining nanoparticles such as Ag-NPs with antibiotics may also enhance antimicrobial activity and possibly subjugate resistance in antibiotics (Huh and Kwon, 2011). In this study, Ag-NPs in the range of 3 – 15 nm in size were produced using the cell free extract of *E. xiangfangensis* Pb204 and evaluated for their antimicrobial activity against ESKAPE pathogens and two other clinically relevant bacterial water-borne pathogens: *V. cholerae* and *E. coli*.

4.3.1 Antibiotic profile of the pathogenic strains

Almost all diseases are treated with antibiotics as the first line drugs to combat and fight infections. However, misusing antibiotics has led to rapid development of resistance genes by many pathogenic strains. In this study, six antibiotics commonly used to treat bacterial infections were selected and tested against eight pathogenic strains of bacteria. Among those eight, six are classified as ESKAPE pathogens known to be the cause of most common infections such as UTIs and life-threatening diseases such as pneumonia. The activity of test antibiotics against individual pathogens was evaluated in triplicate after 24 and 48 hour incubation on Mueller-Hinton agar at 35 ºC. The CLSI guiding document (M100, 27th edition of 2017) was used to classify organisms as susceptible (S), susceptible-dose dependent (SSD), intermediate (I), resistant (I) or nonsusceptible (NS) based on zone diameter value for disk diffusion inhibition. Table 4.1. is an antibiogram detailing the results obtained for each pathogenic strain tested in the study. Where data was unavailable to classify strains as either S, I or R, the discussion of their antibiotic profiles is based on the size of inhibition zones in comparison to those where breakpoints have been identified.

Table 4.1. Antibiogram for the eight bacterial pathogens in this study that are responsible for common infections.[1]

Name of strain	Incubation period (h)	Zones of inhibition (average of triplicates) measured in millimeters (mm)					
		Meropenem (10 µg)	Ampicillin (25 µg)	Cloxacillin (5 µg)	Ciprofloxacin (5 µg)	Vancomycin (30 µg)	Amoxicillin/Clavulanic acid (20/10 µg)
E. faecium	24	0 (R)[2]	27 ± 1.5 (S)	0 (R)	13 ± 0.6 (R)	22 ± 2.0 (S)	30 ± 1.2
	48	0 (R)	29 ± 1.5 (S)	0 (R)	13 ± 1.5 (R)	20 ± 0.6 (S)	28 ± 1.0
S. aureus	24	30 ± 1.5	16 ± 0.6 (R)	0 (R)	25 ± 0.6 (S)	14 ± 0.6	15 ± 1.2
	48	27 ± 0.6	15 ± 1.0 (R)	0 (R)	24 ± 0.6 (S)	15 ± 1.0	13 ± 0.6
K. pneumoniae	24	18 ± 1.7 (R)	0 (R)	0 (R)	19 ± 1.5 (I)	–[3]	10 ± 0 (R)
	48	19 ± 1.5 (R)	0 (R)	0 (R)	18 ± 2.1 (I)	–	10 ± 0 (R)
A. baumannii	24	16 ± 0.6 (I)	32 ± 1.5	0 (R)	16 ± 1.5 (I)	18 ± 1.7	30 ± 1.0
	48	16 ± 1.5 (I)	31 ± 1.2	0 (R)	15 ± 1.2 (R)	18 ± 2.1	30 ± 1.2
P. aeruginosa	24	22 ± 1.2 (S)	0 (R)	0 (R)	30 ± 1.0 (S)	13 ± 1.5	0 (R)
	48	20 ± 1.5 (S)	0 (R)	0 (R)	31 ± 1.5 (S)	0 (R)	0 (R)
E. cloacae	24	21 ± 1.2 (I)	–	0 (R)	18 ± 1.2 (I)	–	17 ± 0.6 (I)
	48	20 ± 1.0 (I)	–	0 (R)	18 ± 0.6 (I)	–	16 ± 0 (I)
E. coli	24	19 ± 1.0 (R)	0 (R)	0 (R)	22 ± 0.6 (S)	–	14 ± 0.6 (I)
	48	29 ± 1.0 (S)	0 (R)	0 (R)	20 ± 0.6 (I)	–	13 ± 0.6 (R)
V. cholerae	24	20 ± 0.6	21 ± 0	0 (R)	30 ± 1.0	–	17 ± 0
	48	21 ± 1.5	21 ± 0	0 (R)	30 ± 2.0	–	17 ± 0.6

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[1] Antibiotics differ in dosage due to availability from manufacturer as well as recommended doses from CLSI.
[2] Strains are classified as susceptible (S), resistant (R) or intermediate (I) based on the diameter of the inhibition zones. Where data was unavailable, strains are not classified as either of the three.
[3] The symbol (−) represents intrinsic resistance of the strains to the test antibiotic.

Horizontal gene transfer and mutations have been known to be the cause of resistance development in ESKAPE pathogens towards most antibiotics. In that regard some antibiotics may not be effective against certain bacteria either due to the dosage or resistance conferred by the bacteria towards those antibiotics. Cloxacillin, a semisynthetic beta-lactam penicillin type antibiotic used in the present study at a dosage of 5 µg, had no antimicrobial activity towards all the strains tested. The antibiotic is commonly used to treat penicillinase-producing staphylococci and penicillin G-sensitive and penicillin G-resistant staphylococci but is ineffective against MRSA with no significant effect on gram negative bacilli. Ampicillin and a combination of amoxicillin/clavulanic acid are also classes of beta-lactam antibiotics used to treat both Gram-positive and Gram-negative bacterial infections. The clavulanic acid is included in the combination antibiotic to restore the efficacy of amoxicillin against bacteria that produce beta-lactamases (Bush and Johnson, 2000; Livermore *et al.*, 2008, Drawz and Bonomo, 2010). Both antibiotics were able to inhibit *E. faecium* and *A. baumannii* while *S. aureus*, *P. aeruginosa* and the three *Enterobacteriaceae* species (*E. cloacae*, *E. coli* and *K. pneumoniae*) exhibited resistance. This is because these species contain resistance genes such as ESBLs and carbapenemases known to be able to destroy chemical structures of beta-lactam antibiotics (Livermore, 2002; Boucher *et al.*, 2009; Santajit and Indrawattana, 2016). The strain of *E. coli* has a similar behaviour to that of *K. pneumoniae* particularly in the zones measured after 24 hours for both the antibiotics. Ramos *et al.* (2014) reported that the genome of *K. pneumoniae* shows the presence of drug efflux pumps in large numbers similar to those observed in *E. coli*, which may explain their similar behavior when exposed to the antibiotics. The two species were also the first to be reported to contain the first mobile colistin resistance gene MCR-1 in China in 2015 (Wang *et al.*, 2018). The resistance by *P. aeruginosa* can also be attributed to ESBL production and the ability to carry other resistance genes such as carbapenemases from *K. pneumoniae* (Santajit and Indrawattana, 2016). Although these antibiotics are not used in the treatment of *V. cholerae*, the zones of inhibition obtained suggest some antimicrobial activity by these antibiotics due to their mode of action.

Meropenem is a beta-lactam carbapenem that is commonly used against bacteria with ESBLs (Mohr III, 2008) and is one of the antibiotics considered to treat *A. baumannii* infections due to its effectivity and safety (Lee *et al.,* 2017). However, *A. baumannii* is a resilient organism that exhibits an exceptional ability to develop antibiotic resistance and as such the emergence of carbapenem-resistant *A. baumannii* (CRAB) has been on the rise (Lee *et al.,* 2017; Isler *et al.,* 2019). The isolate used in the present study exhibited intermediate susceptibility suggesting that a higher dose would be required to prevent its growth. Intermediate susceptibility to the antibiotic was also recorded for *E. cloacae*. *Pseudomonas aeruginosa* and *E. coli* were inhibited by meropenem while *K. pneumoniae* and *E. faecium* showed resistance; no zones of inhibition were observed for *E. faecium*. Considering the large size of

the zones of inhibition (27 mm, 48 h), it may be suggested that meropenem was able to act effectively on *S. aureus* by inhibiting cell wall synthesis.

Antibiotics such as fluoroquinolones exhibit concentration-dependent killing while β-lactams and carbapenems follow time-dependent killing kinetics (Cunha and Opal, 2020), which is one of the aspects that explain differences in antibiotic activity. Ciprofloxacin, a fluoroquinolones class antibiotic that acts by inhibiting DNA replication (Etebu and Arikekpar, 2016) through the inhibition of DNA topoisomerase and DNA gyrase is frequently used to treat UTIs and bacterial pneumonia. It is the most potent fluoroquinolone against gram negative bacilli notably *P. aeruginosa* and *Enterobacteriaceae* like *E. coli* (Grillon *et al.,* 2016). In agreement with this observation, both *P. aeruginosa* and *E. coli* were found to be susceptible to the antibiotic while the other members of *Enterobacteriaceae* (*E. cloacae* and *K. pneumonia*) exhibited intermediate susceptibility using a dose of 5 µg ciprofloxacin. Ciprofloxacin is also known to have some effectiveness against gram positives such as was evident for *S. aureus* in this study where a zone of inhibition of 24 mm (48 h) was observed. However, it was ineffective against *E. faecium*.

Vancomycin is commonly used in the treatment of MRSA and enterococci. From table 4.1, it is evident that *E. faecium* is susceptible to a dose of 30 µg of vancomycin. Exposure of *S. aureus* to vancomycin resulted in a zone of inhibition that measured 14 mm after 24 hours and 15 mm after 48 hours. The CLSI recommends that MIC tests are preferable for determining the susceptibility of staphylococci to vancomycin because the disk test is inefficient at differentiating between vancomycin-susceptible and vancomycin-intermediate isolates of *S. aureus*. Vancomycin resistant *S. aureus* (VRSA) carry the *VanA* resistance gene and do not show a zone of inhibition around the disk (zone = 6 mm) and their identity should be confirmed. Alternatively, isolates that show zones of inhibition ≥15 mm may be considered susceptible but require further confirmation using an MIC test as they could potentially be vancomycin intermediate *S. aureus* (VISA) strains (https://www.cdc.gov/hai/settings/lab/visa_vrsa_lab_detection.html#:~:text=Most%20isolates%20of%20S.,In%20contrast%2C%20S). Based on this information, it is probable that the isolate used in this study belongs to VISA. *Acinetobacter baumannii* species are intrinsically resistant to vancomycin due to the large nature of glycopeptide molecules that cannot penetrate their outer membrane. Interestingly, a zone of inhibition of 18 mm resulted from the exposure of *A. baumannii* to vancomycin in this study. A definitive conclusion cannot be made as to whether this isolate is susceptible or resistant to vancomycin although at present, vancomycin is used in combination with meropenem and colistin/polymyxin to treat MDR *A. baumannii* strains (Wang *et al.,* 2019; Shinohara *et al.,* 2019).

The overall findings from the antibiotic profiles suggest that at least one of the antibiotics tested in this study exhibits acceptable antimicrobial activity against

each ESKAPE pathogen. However, these isolates exhibit MDR to two or more of the antibiotics tested. *Escherichia coli* was susceptible to meropenem and ciprofloxacin but was able to resist the penicillin-type antibiotics. While the CLSI does not report the breakpoints for *V. cholerae* with any of the antibiotics tested, the zones of inhibition suggest that it could be susceptible to some beta-lactams and ciprofloxacin but cloxacillin is unable to inhibit its growth. Moreover, cloxacillin was the least effective antibiotic as all eight bacterial pathogens were resistant to it. Both vancomycin and some penicillins were able to inhibit the growth of the Gram-positive isolates albeit without a distinct pattern. No conclusion can be made with regards to the effectiveness of one antibiotic over another in treating the Gram-negatives.

4.3.2 Antimicrobial activity of Ag-NPs on the pathogenic strains

With the use of an xMark Microplate spectrophotometer antimicrobial activity of these Ag-NPs was observed. The Ag-NPs appear to be bacteriostatic particularly those at 0.5 and 1.0 mM as compared to those at 0.1 and 0.25 mM which exerted little to no inhibition on the growth of all the strains tested. As already reported in many studies Ag-NPs possess inhibitory effects against many microbial agents including viruses, fungi, yeasts and bacteria. Figure 4.2 below represents the antimicrobial activities of Ag-NPs at 0.1, 0.25, 0.5 and 1.0 mM concentrations against eight bacterial strains that included six ESKAPE pathogens plus *E. coli* and *V. cholerae*.

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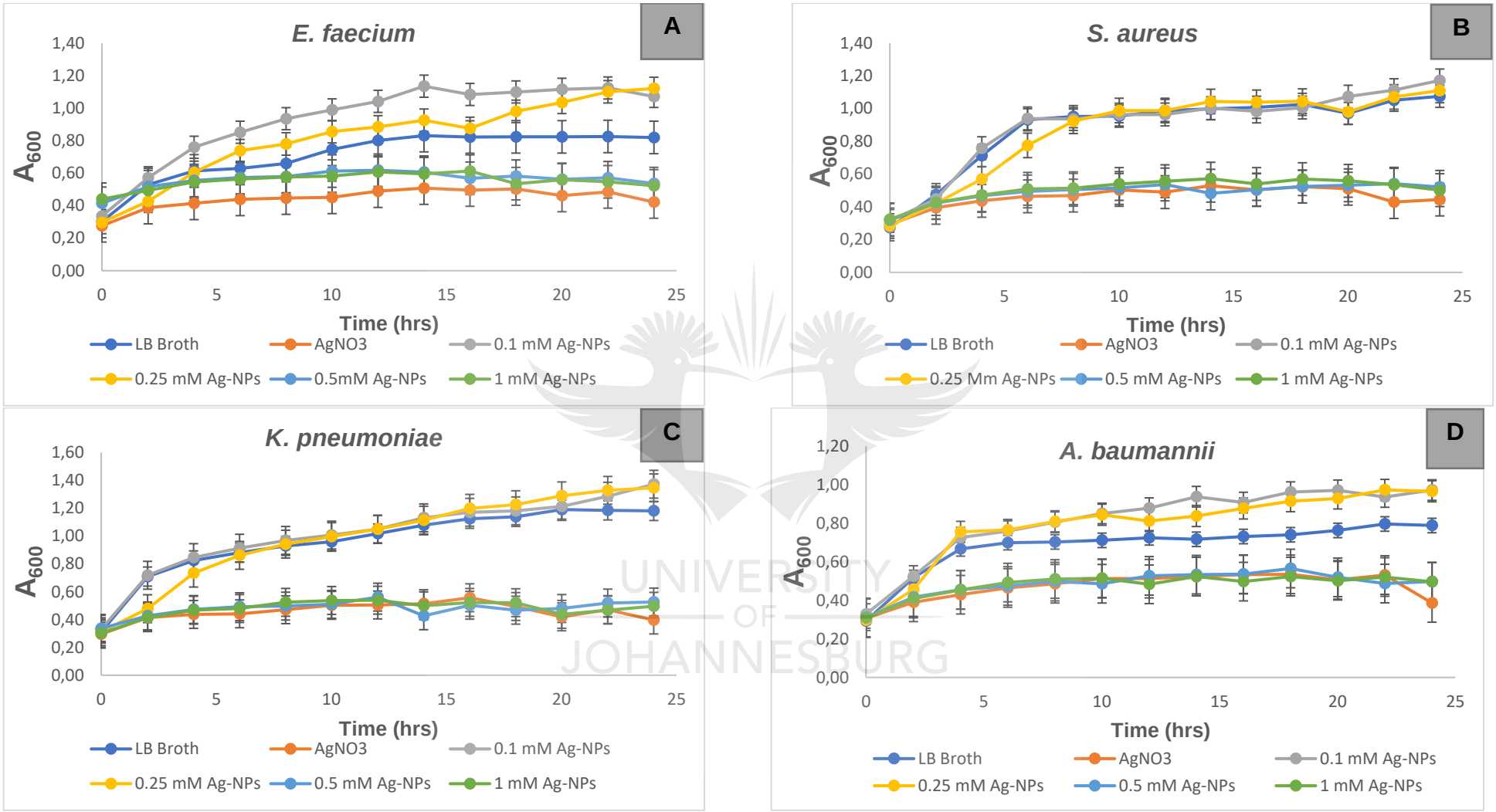

Figure 4.2. Antimicrobial activity of $AgNO_3$ and Ag-NPs on eight bacterial strains, with six strains classified as ESKAPE pathogens plus *E. coli* and *V. cholerae*. Each graph represents antimicrobial activity of Ag-NPs against; (A) *E. faecium*, (B) *S. aureus*, (C) *K. pneumoniae*, (D) *A. baumannii*, (E) *P. aeruginosa*, (F) *E. cloacae*, (G) *E. coli* and (H) *V. cholerae*. The $AgNO_3$ at 1.0 mM and Ag-NPs at 0.5 and 1.0 mM are able to limit the microbial activity to a lower growth rate compared to those at 0.1 and 0.25 mM. Figure 4.2 continued on page 43.

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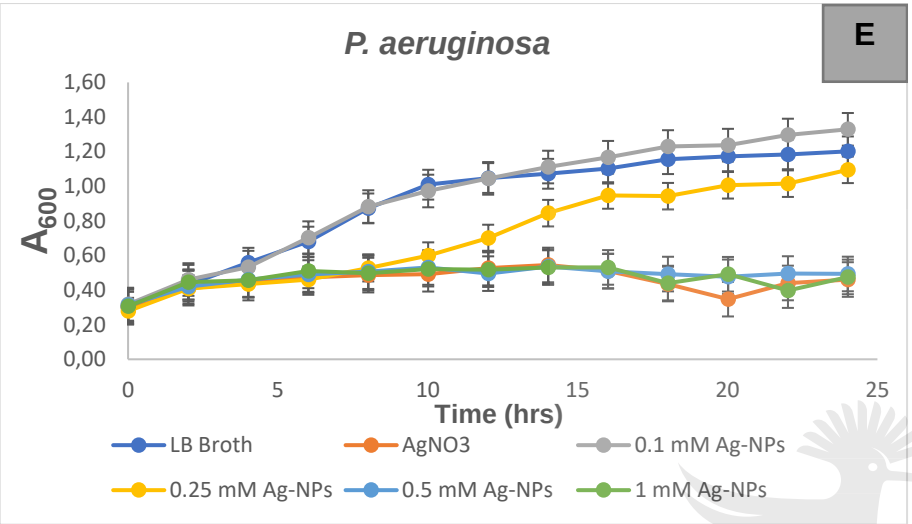

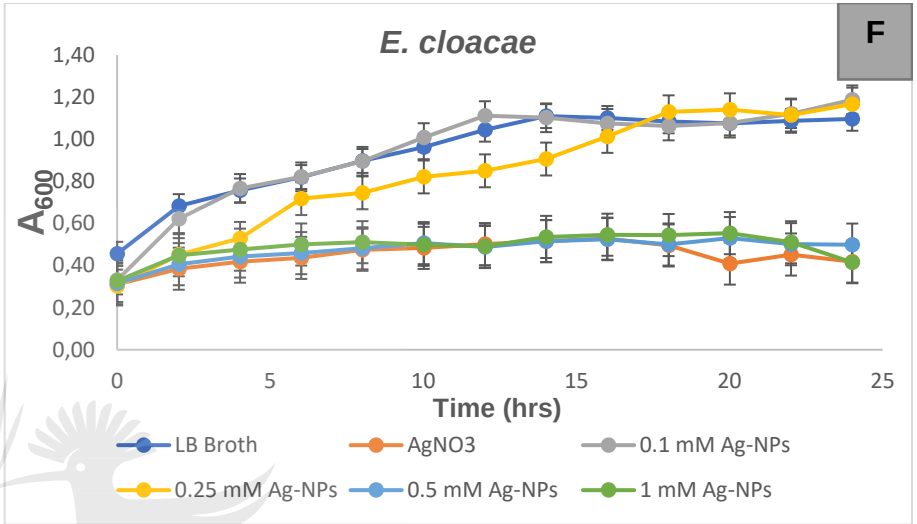

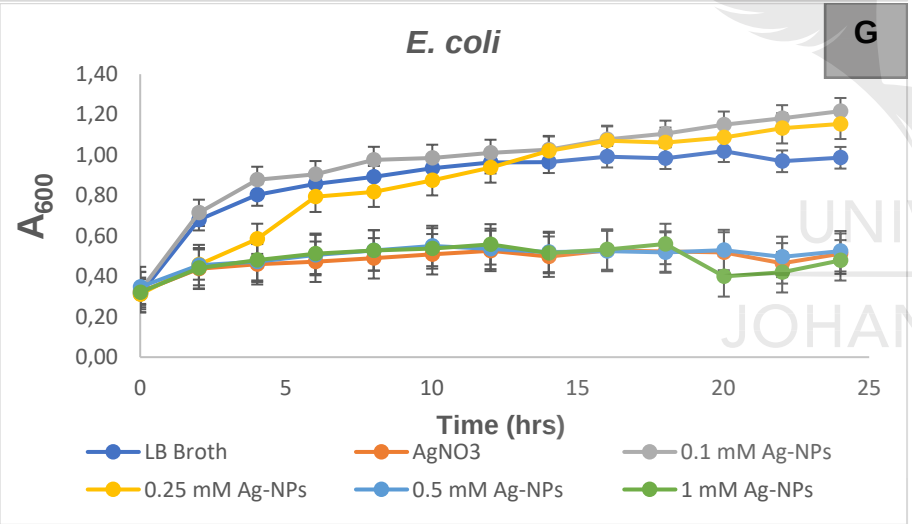

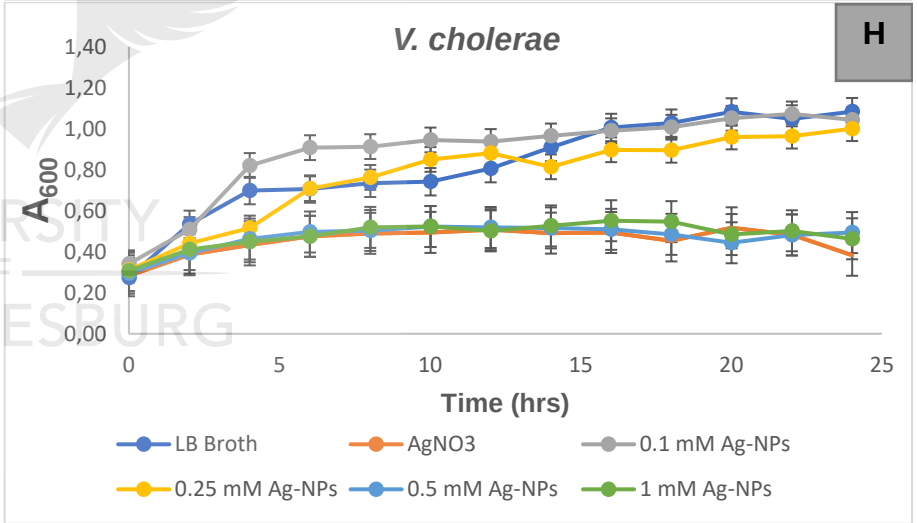

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From figure 4.2, it can be observed that both 1 mM $AgNO_3$ and Ag-NPs exert an inhibitory effect on bacterial growth; but the antimicrobial activity of Ag-NPs is dependent on the dosage/concentration. Silver nanoparticles at 0.1 mM (10.8 µg/ml) and 0.25 mM (27 µg/ml) concentrations were unable to inhibit microbial growth of all the bacterial strains. However, the growth rate of all the strains, except for *E. faecium* and *A. baumannii*, in 0.25 mM Ag-NPs is initially slower than that in 0.1 mM Ag-NPs and the control (without Ag), as indicated by the exponential growth phase in the growth curves. The longer exponential phase in these species at 0.25 mM Ag-NPs compensates for the slower growth rate such that by the time stationary phase is reached, the bacterial numbers are similar to the control (without Ag) and 0.1 mM Ag-NP cultures. Only *P. aeruginosa* and *V. cholerae* experienced slightly decreased growth. In the case of *E. faecium* and *A. baumannii*, both bacteria seemed to favour growth in the presence of 0.1 mM and 0.25 mM Ag-NPs with increased numbers compared to the LB control that did not contain any Ag. This was unexpected as Ag-NPs have been reported to be effective against these strains and their resistant derivatives (Khan *et al.*, 2019). However, there was lower growth of *E. faecium* in the presence of 0.25 mM compared to 0.1 mM Ag-NPs which was not observed for *A. baumannii*.

In contrast to 0.1 and 0.25 mM concentrations, Ag-NPs at 0.5 (53.9 µg/ml) and 1.0 mM (107.85 µg/ml) concentrations limited the microbial growth significantly for all 8 pathogenic strains. An initial increase (~double) in the A_{600} over the first 2.5 hours of incubation is evident indicating some replication of bacterial cells occurred. However, continued culturing did not result in increased numbers but rather a plateau in the growth curves pointing to the Ag-NPs having a bacteriostatic effect on these eight pathogenic strains. A similar trend was observed when the pathogens were grown in the presence of 1 mM $AgNO_3$. Of all eight pathogenic strains, *E. faecium* behaved somewhat differently to the others whereby the growth achieved in the presence of 0.5 and 1 mM Ag-NPs was slightly higher than in the presence of 1 mM $AgNO_3$. This could be an indication that 1 mM $AgNO_3$ has a better inhibitory effect than the Ag-NPs on *E. faecium*.

In a similar study to the current one, MICs of Ag-NPs were determined to be 3.125 µg/ml, 1.56 µg/ml and 3.125 µg/ml against *E. coli*, *P. aeruginosa* and *K. pneumoniae*, respectively (Shaker and Shaaban, 2017). Krishnaraj *et al.* (2010), tested Ag-NPs biosynthesized using leaf extracts of *Acalypha indica* against *E. coli* and *V. cholerae*. Their study established an MIC of 10 µg/ml Ag-NPs (size range of 20 –30 nm) for *E. coli* and *V. cholerae*. The concentration of Ag-NPs found to inhibit growth of *E. coli* and *V. cholerae* in the present study were at a concentration of 53.9 µg/ml (0.5 mM) and 107.85 µg/ml (1 mM) for spherical Ag-NPs ranging between 3 – 15 nm in size which is higher than the values reported in these studies. The same concentration of Ag-NPs also inhibited the growth of the six ESKAPE pathogens. The differences in inhibitory concentrations can be attributed to size of the Ag-NPs as well as medium-

related factors due to silver binding components such as microbial biomass and proteins (Zhang *et al.*, 2014). In addition, the different inoculum sizes used to determine MICs in this study and other studies also contributes to the differences observed during antimicrobial activities. For example, Shaker and Shaaban (2017) used starting cultures at a concentration of 5×10^6 cells/ml compared to this study which used ~ 5×10^8 cells/ml.

Silver solutions have been used since the early 20th century as an antimicrobial agent (Concepcion *et al.*, 2007). Utilizing silver as an antimicrobial agent in burn care has also been reported (Castellano *et al.*, 2007; Li *et al.*, 2015; Munteanu *et al.*, 2016). Ross *et al*. (2020) demonstrated that nanocrystalline silver-based wound dressing had higher antimicrobial activity than high-oxidation silver salts and silver-plated nylon. Silver nanoparticles can induce higher toxic effects according to the concentration of dissolved silver (Navarro *et al.*, 2008), presumably due to additional effects of particles and agglomeration on cell membranes (Lapresta-Fernández *et al.*, 2012), which depends upon factors such as media used (Oukarroum *et al.*, 2012), light conditions, organic molecules and particle size or nanoparticle coating (Liu *et al.*, 2010; Shi *et al.*, 2012). Silver-containing materials have been studied widely due to antimicrobial activity of silver on bacteria, viruses and fungi (Banerjee *et al.*, 2011; Liu *et al.*, 2013; Agnihotri *et al.*, 2014). However, there are concerns on the use of bulk silver that may result in the same fate as antibiotics if not checked. Beyond the extensive antimicrobial properties of Ag-NPs, using them reduces the bulk concentration of silver subsequently avoiding development of resistance by microorganisms to silver. Moreover, biologically synthesized Ag-NPs offer greater antibacterial effects compared to those synthesized using chemical methods.

Biosynthesized Ag-NPs displayed greater antimicrobial effects against *S. typhi*, *B. cereus* and *P. aeruginosa* compared to chemically synthesized Ag-NPs (Chahar *et al.*, 2018). Similar observations were made by Antony *et al.* (2011) when they studied the effects of biosynthesized and chemical synthesized Ag-NPs against *S. aureus*, *K. pneumoniae*, *B. subtilis*, *E. coli*, *Proteus vulgaris*, *P. aeruginosa* and *S. typhi*. Concerns have been raised with regards to the overuse of Ag^+ and Ag-NPs which may lead to silver resistance (Percival *et al.*, 2005; Randall *et al.*, 2015), promote antibiotic resistance via mutations (Kaweeteerawat *et al.*, 2017) or horizontal transfer of antibiotic resistance genes (Lu *et al.*, 2020).

Taking into consideration the highest dosage of an antibiotic that an individual pathogen was susceptible to, the findings reveal that for some bacterial species a higher dosage of the specific antibiotic (20-30 µg, section 4.3.1) is needed to inhibit growth compared to the concentration of Ag-NPs that were needed to yield similar results. Inhibition of growth was achieved at a minimum dosage of 10.79 µg Ag-NPs. This was true for four out of the eight pathogens tested: *E. faecium*, *A. baumannii*, *E. cloacae* and possibly *V. cholerae*.

4.4 Conclusion

The chapter aimed to establish the antimicrobial activity of Ag-NPs biosynthesized by *E. xiangfangensis* Pb204 and compare it to that of commonly used antibiotics to treat clinically important bacterial pathogens. Reports from previous studies describe the antimicrobial effect of Ag-NPs against a wide range of microorganisms including MDR bacteria that are able to resist antibiotics. Biogenic spherical Ag-NPs (produced in this study) ranging in size from 3 – 15 nm were effective at inhibiting the growth of all eight pathogens at a MIC of 53.9 µg/ml. Moreover, the inhibitory effect was independent of the type of pathogenic strain tested against unlike the antibiotics where individual bacterial species are only successfully inhibited by a specific antibiotic(s). The outcome of whether the antimicrobial activity of Ag-NPs surpasses that of antibiotics used in the treatment of the pathogens tested here is inconclusive. Nonetheless, it is noteworthy to mention that three of the ESKAPE pathogens (*E. faecium*, *A baumannii* and *E. cloacae*) as well as *V. cholerae* responded better to the treatment with Ag-NPs in terms of µg dosage than antibiotics. The use of these Ag-NPs as an alternative treatment for clinically important bacterial infections is promising in terms of lowering the mortality rate, reducing the cost invested in antibiotics which is a heavy burden on the health sector and the continued rise in antibiotic resistant microorganisms.

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Chapter 5: General conclusion and recommendations

The ability of *E. xiangfangensis* Pb204 to reduce heavy metals to nanoparticles is due to ICEs which codes for proteins involved in various metal resistance including silver. Silver has been used as an antimicrobial since the early 20th century. With the rise in antimicrobial resistance, silver has played a pivotal role in nanoscience lately, from development of silver-containing materials to synthesis of nanoparticles. The prevalence of AMR is a tragic phenomenon that has resulted in high mortality rate, staggering healthcare expenditure and many uncertainties in the world. This research falls among other studies carried out to support the use biologically synthesized Ag-NPs as an alternative therapy for the treatment of common yet harmful bacterial infections that are exacerbated due to AMR. The advantage of biological methods is the use of nontoxic molecules such as proteins, naturally secreted by microorganisms compared to toxic chemical stabilizers and reducing agents used in chemical processes which have negative effects in the environment.

To the best of our knowledge this is the first study that has attempted to optimize the reaction parameters for Ag-NPs synthesis by *E. xiangfangensis* Pb204 which were tested for their antimicrobial activity against common clinically important pathogens. In an effort to reproducibly produce uniformly superior and effective Ag-NPs intended for use as antimicrobials, several reaction parameters that included pH, temperature and reaction time were optimized. Optimisation of the reaction parameters to pH 7, temperature at 37 ºC and short incubation period of 24 hours during Ag-NP synthesis by *E. xiangfangensis* Pb204 resulted in a high yield of uniformly spherical particles between 3 − 15 nm in size with a good distribution. At a MIC of 53.9 µg/ml (0.5 mM), these Ag-NPs were able to inhibit the growth of eight pathogenic strains which included six ESKAPE pathogens known for their resistance capacity. The antimicrobial activity of Ag-NPs is attributed to their ability to damage and penetrate cell walls of microorganisms, followed by the release of ROS that destroys the cells nucleic acids and proteins.

Antibiotics are used as first line treatment for almost all infections, which is alarming considering the prevalence of AMR against these antibiotics. Five out of the six antibiotics were able to inhibit the growth of the bacterial pathogens, although some strains were resistant to these antibiotics. For instance, *K. pneumoniae* was resistant to both meropenem (10 µg) and amoxicillin/clavulanic acid (20/10 µg). Ciprofloxacin (5 µg) was the most effective antibiotic considering its dosage even though *E. faecium* and *A. baumannii* were resistant to the antibiotic after 48- hour incubation period. These antibiotics are used to treat common infections including hospital acquired infections such as UTIs, meningitis, pneumonia and diarrhea. It is a concern when they are ineffective leading to treatment with more severe drugs that are relatively expensive. Antimicrobial resistance is growing at an alarming rate, already leading to human mortality, GDP, poverty, healthcare costs and livestock output. It is anticipated that by 2050 the GDP and world trade will both fall by 1.1% and 3.8% in low impact and high impact AMR scenario, respectively (The World Bank, 2017). As explained by Ahmad and Khan (2019), if AMR is

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ignored, it may lead to medical poverty trap due to its high cost on the population and economy.

Silver nanoparticles in the study exhibited similar and consistent antimicrobial activity for all eight bacterial pathogens at a MIC of 0.5 mM (10.79 µg, dosage). On the other hand, the antibiotics were strain dependent, implying that a specific antibiotic will work on specific pathogen or a group of pathogens such as *Enterobacteriaceae*. Normally antibiotics are designed to inhibit the growth of certain microorganisms which explains the above observation. For instance, ampicillin is one of the major active beta-lactam antibiotics against enterococci, able to inhibit the synthesis of its peptidoglycan (Miller *et al.*, 2014). In addition, the findings from the study provided evidence of developing resistance in these pathogens. At 25 µg dosage, ampicillin was able to inhibit the growth of *E. faecium* whereas *S. aureus* was resistant to the antibiotic. Furthermore, the dosage of this antibiotic was high (25 µg) compared to 10.79 µg of Ag-NPs used to inhibit bacterial growth. In a case of resistant *E. faecium*, particularly VRE, the combination of daptomycin and ampicillin have been reported to be effective (Sakoulas *et al.*, 2012); however, these measures increase the costs associated with treatment.

Alternatively, the use of Ag-NPs in treatment of these pathogens may be implemented. In comparison of the two antimicrobials, this study showed that for some but not all pathogenic strains tested Ag-NPs are more effective at lower dosage compared to the antibiotics. The effect of these nanoparticles is in line with many studies conducted to test the antimicrobial of Ag-NPs on various microorganisms which were successfully suppressed by Ag-NPs at lower concentrations.

The study did have some limitations in terms of the methodologies used that may have influenced the outcomes reported here. Microorganisms produce distinct enzymes in different quantities which can exert an effect on the rate of nanoparticle synthesis (Haverkamp *et al.*, 2007) and therefore the yield when involved in these types of biogenic pathways. In addition, control of biological synthesis is more challenging in terms of uniformity and homogeneity when compared to chemical synthesis. This could have contributed to size variations (in different ratios) each time nanoparticles were prepared even though the conditions of synthesis were for the most part optimised. This can have a slight effect on antimicrobial properties per batch synthesised. Often sedimentation by standard centrifugation can lead to some loss of nanoparticles and does not differentiate between different shaped nanoparticles in mixtures. This can be limited by using sucrose gradients and high speed centrifugation to separate mixtures of nanoparticles into their distinct shapes while concentrating them. Disc diffusion method was initially used to test the antimicrobial activity of the Ag-NPs with little success. This may be because of the poor diffusion of Ag-NPs in solid culture media. Nanoparticles do not travel far from the disc to physically interact with the bacterial cells (Kourmouli *et al.*, 2018), which may explain the lack of inhibition zones observed during the antimicrobial test. To circumvent this limitation, growth curves in the presence of Ag-NP-containing

culture broth were conducted to evaluate their antimicrobial activity. This method of broth dilution is in fact a more accurate technique to establish MICs for any antimicrobial agent. Furthermore, a true comparison of Ag-NPs versus antibiotics was not feasible due to the difference in methodology used. It is therefore recommended that the study be repeated using the dilution method to establish the MICs for the antibiotics and that it is extended to evaluate the combined use of Ag-NPs and antibiotics in the treatment of these pathogens.

The findings reported here support the continued pursuit of these Ag-NPs as an alternative to antibiotic treatment. Using the optimised reaction parameters, the production of these Ag-NPs can be scaled up and the knowledge gained implemented in the design of an industrial process for their large-scale production. Narrowing the MIC for the Ag-NPs can be performed to establish an even lower dosage required to prevent growth of the pathogens while more accurate broth MICs can be determined for the antibiotics. Since there is no evidence supporting the superiority of Ag-NPs over antibiotics or vice versa, it is hereby suggested that further testing of the combined use of Ag-NPs with antibiotics is advantageous for treating ESKAPE pathogens. This treatment strategy could result in enhanced antimicrobial activity, reduced AMR and more effective treatment especially against antibiotic resistant strains.

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